Chapter 5
Mechanisms of Action of Bacterial Biological Control Agents

The existence of natural suppressive soils has been recognized in the Salinas valley (California, United States), the Chateaurenard region near Cavaillon (France), the Canary Islands and the Broye Valley (Switzerland). Crops grown on suppressive soils do not suffer much from certain disease(s), due to reduction in disease incidence and/or severity, in spite of the presence of the pathogen(s) in those soils. In contrast, the conducive soils allow the development of the pathogens and the diseases induced by them. The phenomenon of disease suppression in certain soils showed two common characteristics: (i) loss of suppressiveness, when the soil was pasteurized and (ii) transferability of suppressiveness to conducive soils (opposite of suppressiveness). These two characteristics indicated the involvement of the activities of soil microorganisms that are sensitive to heat or other adverse conditions. The suppressiveness of the soil associated with the presence of different species of bacteria and fungi may be predominantly, due to the production of various kinds of antimicrobial compounds, in addition to aggressive colonization of available plant surfaces, competition for nutrients and stimulation of natural plant defense systems. Some of the microorganisms have been shown to promote the growth of the treated plants. The various mechanisms of the biocontrol activities of plant-associated bacterial species are discussed in this chapter.

The bacterial biocontrol agents (BCAs) may act directly or indirectly by different mechanisms such as antibiosis, competition for nutrients, colonization of specific sites required for establishing infection by the pathogens and inducing resistance to the pathogens by activating host plant defense systems. Furthermore, the plant growth-promoting rhizobacteria (PGPR) are known to enhance plant growth, in addition to the protection of the plants against microbial plant pathogens. The mechanisms of biocontrol activities of plant-associated bacterial species are discussed to have an insight into the three-way interactions among plants, bacterial biocontrol agents and plant pathogens.

P. Narayanasamy, *Biological Management of Diseases of Crops*,
Progress in Biological Control 15, DOI 10.1007/978-94-007-6380-7_5,

5.1 Types of Antagonism

Antagonism exhibited by bacterial biocontrol agents predominantly depends on the ability to produce compounds that are inhibitory to bacterial and fungal plant pathogens infecting various crops or adversely affect the pathogen development in other ways (Table 5.1).

5.1.1 Pseudomonas *spp.*

5.1.1.1 Metabolites-Mediated Antagonism

Antibiotics

Root colonization is a process by which bacteria introduced on seeds, vegetatively propagated plant parts, or into soil are distributed along roots. Plant-associated bacteria have the ability to colonize and persist in the rhizosphere and they are collectively called as rhizobacteria. The plant growth-promoting rhizobacteria (PGPR) form an important group of bacterial biocontrol agents that are being employed for the management of crop diseases in various ecosystems. The PGPR species are classified under the genera *Pseudomonas, Bacillus, Azospirillum, Rhizobium* and *Serratia*. *Pseudomonas* strains with antagonistic potential play clear colony phase variation, exhibiting opaque and translucent colonies (Phase I). The important biocontrol traits such as motility and production of antifungal metabolites, proteases, lipases, chitinases and biosurfactants are correlated with phase I morphology and these characteristics are absent in bacteria with phase II morphology. Phase variation is based on structural changes at the DNA level. Phase variation as a regulatory system can influence the production of diverse traits such as production of proteases and lipases, pili, outer membrane proteins, fimbriae and surface lipoproteins (van den Broek et al. 2003). Although the PGPRs have multiple mechanisms of biocontrol activities, production of different kinds of antibiotics seems to be the principal mechanism of action against crop pathogens. These BCAs secrete phloroglucinols, phenazines, pyoluteorin, pyrrolnitrin, rhamnolipids. Several antibiotics of lesser importance are also produced by the different genera of PGPR. The activities of these metabolites against microbial pathogens are described.

Production of antibiotics by bacterial species with biocontrol potential is demonstrated in five steps. Diffusible or volatile secondary metabolites secreted by bacterial strains in vitro are purified and identified chemically. Then the antibiotic compound of significance is detected and quantified in the rhizosphere or natural substrates into which the putative bacterial BCA is introduced. The HPLC process of purification has been applied for this purpose. The structural and the principal regulatory genes controlling the expression of the antibiotic compound are identified and characterized. In the case of intrinsically poor biocontrol strains, they can be transformed by

Table 5.1 Production of antibiotics by bacterial biocontrol agents effective against microbial plant pathogens

Bacterial biocontrol agent/ antibiotics produced	Microbial plant pathogens	References
I. *Pseudomonas* spp.		
A. Pholoroglucinols 2,4-diacetylphloro-glucinol (2,4-DAPG)	*Gaeumannomyces graminis* var. *tritici*	Raaijmakers and Weller (1998) and de Werra et al. (2009)
	Fusarium oxysporum f.sp. *pisi*	Landa et al. (2002)
	Pythium ultimum var. *sporangiferum*	de Souza et al. (2003a, b)
	Rhizoctonia solani and *sclerotium rolfsii*	De La Fuente et al. (2004)
	Aphanomyces cochlioides	Bechewich and Hearth (1998) and Islam and Fukushi (2010)
	Xanthomonas oryzae pv. *oryzae*	Velusamy et al. (2006)
B. Pyoluteorin	*Pythium ultimum*	Howell and Stipanovic (1980)
	Rhizoctonia solani	Brodhagen et al. (2004)
C. Pyrrolnitrin	*Botrytis cinerea* *Gaeumannomyces graminis* var. *tritici*	Hammer and Evensen (1993), Tazawa et al. (2000), and Ajouz et al. (2009)
D. Aerugine	*Colletotrichum orbiculare* *Phytophthora capsici* and *Pythium ultimum*	Lee et al. (2003)
E Phenazines	*Gaeumannomyces graminis* var. *tritici*	Thomashow et al. (1990) and Pierson and Thomashow (1992)
	Fusarium oxysporum f.sp. *ciceris* and *F. udum*	Anjaiah et al. (2003)
	Fusarium oxysporum f.sp. *radicis-lycopersici*	Chin-A-Woeng et al. (2001a, b)
	Rhizoctonia solani	Rosales et al. (1995)
	Verticillium longisporum	Debode et al. (2007)
	Pythium myriotylum, P. spendens	Perneel et al. (2007, 2008)
	Sclerotinia sclerotiorum	Athukorala et al. (2010)
II. *Bacillus* spp.		
A. Surfactins	*Sclerotinia sclerotiorum* *Fusarium graminearum*	Athukorala et al. (2009)
B. Bacillomycin D	*S. sclerotiroum*	Athukorala et al. (2009)
	F. graminearum	
	Alternaria alteranta *Fusarium graminearum*	Zhao et al. (2010)
C. Fengycins	*S. sclerotiroum*	Athukorala et al. (2009)
	F. graminearum	
D. Azalomycin F	*Xanthomonas campestris* pv. *campestris*	Kim et al. (2003)
	Botrytis cinerea	
	Fusarium oxysporum	
	Pythium sp.	
	Rhizoctonia solani	

(continued)

Table 5.1 (continued)

Bacterial biocontrol agent/ antibiotics produced	Microbial plant pathogens	References
D. Iturins	*S. sclerotiroum*	Athukorala et al. (2009)
	F. graminearum	
	Colletotrichum truncatum	Huang et al. (2005)
	Macrophomina phaseolina	
	Phomopsis sp.	
	Rhizoctonia solani Sclerotinia sclerotiorum	
	Botrytis cinerea	Pyoung et al. (2010)
	Fusarium solani	
	Rhizoctonia solani	
	Phytophthora capsici	
III. *Paenibacillus* spp.		
Polymyxins	*Pseudomonas* spp.	Wiese et al. (1998)
Antifungal peptides	*Rhizoctonia solani*	Chen et al. (2010)
IV. *Burkholderia*		
Pyrrolnitrin	*Rhizoctonia solani*	Lievens et al. (1989)
Pyoluteorin		Baligh et al. (1999)
V. *Serratia*		
Pyrrolnitrin	*Sclerotinia sclerotiorum*	Rite et al. (2002)
Prodigiosin	*Penicillium expansum*	Levenfors et al. (2004)
VI. *Pantoea*		
Herbicolins	*Erwinia amylovora*	Ishmaru et al. (1988)
Pantocins		Wright et al. (2001)
VII. *Streptomyces* spp.		
Geldanamycin	*Rhizoctonia solani*	Beauséjour et al. (2001)

integrating the desired antibiotic biosynthetic genes that are not present in the original strains. Finally, the expression of the desired antibiotic biosynthetic genes has to be demonstrated in the rhizosphere through the use of easily detectable reporter genes that are fused to the structural genes for antibiotic biosynthesis (Haas and Défago 2005).

Fluorescent *Pseudomonas* spp. are found in abundance in the rhizosphere of plants and their ability to suppress the development of fungal and bacterial pathogens and the diseases induced by them in a wide range of crops in different ecosystem has been demonstrated. In addition, the pseudomonads exert beneficial stimulatory effect on plant growth, resulting in increased yield of agricultural and horticultural produce. These bacterial species have been intensively studied, because of the benefits provided to crop cultivation. These bacterial species secrete several antibiotics, siderophores and hydrogen cyanide (HCN) which are involved in their biocontrol activities. The antibiotics produced by *Pseudomonas* spp. belong to six classes, phloroglucinols, phenazines, pylouteorin, pyrrolnitrin, cyclic lipopeptides (all of which are diffusible) and volatile hydrogen cyanide (HCN). The complete genomic analysis to determine the sequences involved in different activities of bacterial biocontrol agents including the biosynthesis of various secondary metabolites has

been performed. In *Pseudomonas fluorescens* strain Pf-5, nearly 6 % of the 7.07 Mb genome is devoted to the biosynthesis of secondary metabolites, including antibiotics toxic to soilborne fungi and oomycetes that infect plant roots and two siderophores involved in iron acquisition. Three orphan gene clusters, for which the encoded natural product was not known earlier, were also identified in the genome of Pf-5 strain. The product synthesized from one of the orphan clusters was identified using the genomesotopic approach, employing a combination of genomic sequence analysis and isotope guided fractionation. One of the orphan gene clusters in Pf-5 was shown to be involved in the biosynthesis of orfamide A, member of a new group of bioactive cyclic lipopeptides with a putative role in biological control of microbial pathogens, causing plant diseases (Loper and Gross 2007).

Pholoroglucinols

Among the phloroglucinols, 2,4-diacetylphloroglucinol (2,4-DAPG) is well known. This phloroglucinol PhlD is a polyketide responsible for the production of monoacetyl phloroglucinol (MAPG) and PhlA, PhlC, and PhlB are required to convert MAPG to 2,4-DAPG (Bangera and Thomaslow 1999). Two major phenotypic groups of 2,4-DAPG producers have been distinguished based on the production of antifungal compounds: one group can synthesize 2,4-DAPG, HCN and pyoluteorin and another group synthesizes only 2,4-DAPG and HCN. The abundance of 2,4-DAPG-producing *Pseudomonas* spp. has been correlated with the natural suppression of wheat take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Raaijmakers and Weller 1998). The fluorescent pseudomonads form an important group of plant growth-promoting rhizobacteria (PGPR) involved in the biological control of several crop diseases. They suppress the development of root and seedling diseases. *Pseudomonas fluorescens* CHA0 suppressed tobacco black root disease (Stutz et al. 1986), wheat take-all disease (Keel et al. 1992) and tomato wilt and crown and root rot disease (Tamietti et al. 1993; Duffy and Défago 1997). Suppression of sugar beet damping-off disease by *Pseudomonas* spp. (Shanahan et al. 1992) and wheat take-all disease by *P. fluorescens* strains Q2-87 and Q8rl-96 (Pierson and Weller 1994; Raaijmakers and Weller 1998) was also demonstrated. Although much progress has been made in the understanding of the biosynthesis and regulation of 2,4-DAPG production by *Pseudomonas* strains, adequate attention does not appear to have been paid to investigate the responses of fungal pathogens to 2,4-DAPG and the mode of its action on the target pathogens. Mostly, the effect of 2,4-DAPG was assessed on the mycelial growth. The fungal pathogens are known to produce different spore forms and structures that are resistant to chemicals and other adverse environmental conditions. Hence, it is essential to understand the variations in the sensitivity of different phases of the life cycle of the pathogen to a specific biocontrol trait for assessing the potential of the biocontrol agents possessing such traits.

Inconsistent performance of most bacterial biocontrol agents under field conditions from site to site and from year to year tends to be the disappointing reality. To overcome this obstacle, it is necessary to understand the sources of variability in the BCA performance. *Pseudomonas fluorescens* CHA0 produces the antibiotic

2,4-diacetylphloroglucinol (2,4-DAPG) that has a key role in its mechanism of biocontrol activity. The strain CHA0 carrying a translational *phlA'-lac* fusion was used to monitor the expression of the *phl* biosynthetic genes governing 2,4-DAPG production in vitro and in the rhizosphere. The reporter gene expression was greater in the rhizosphere of maize and wheat (monocots) compared with the gene expression in bean and cucumber (dicots). Similar variations were observed at cultivar levels also. Plant age had significant impact on gene expression. Root infection by *Pythium ultimum* stimulated bacterial gene expression on both cucumber and maize and this was dependent on differences in rhizosphere colonization on these plant species. The enhanced gene expression may probably be attributed to an indirect effect of disease (causing greater release of root exudates) rather than to a direct signaling from the pathogen (Notz et al. 2001).

Strains of *Pseudomonas fluorescens* produce DAPG that could inhibit growth of diverse group of different groups of organisms including fungi, bacteria, protists and nematodes (Bender and Rangaswamy 1999). The responses of *Pythium* spp., a ubiquitous pathogen infecting several crops to 2,4-DAPG were studied. The variation in sensitivity of 14 *Pythium* isolates obtained from multiple hosts to 2,4-DAPG was assessed. Variation within and among *Pythium* spp. to 2,4-DAPG was observed. Different propagules of *P. ultimum* var. *sporangiferum* exhibited significant differences in their sensitivity to 2,4-DAPG. Zoospores were the most sensitive, followed by zoosporangia, the mycelium being the most resistant structure. The activity of 2,4-DAPG was influenced by pH levels and the low pH favored its activity. Level of acetylation of phloroglucinols was found to be a major determinant of their activity. Ultrastructural alterations in hyphal tips of *P. ultimum* var. *sporangiferum* exposed to 2,4-DAPG were assessed, using the transmission electron microscope (TEM). Different stages of disorganization in hyphal tips of the pathogen were observed. Localized alteration (proliferation or disruption) in plasma membrane organization, development of an extensive network of smooth membranous vesicles, degenerated cytoplasm bordered by a retracted plasma membrane and hyphal senescence accompanied by vacuolization and degeneration of its content were frequently observed alterations in the fungal pathogen. It appeared that 2,4-DAPG did not affect the cell wall structure and composition of hyphal tips of the pathogen, since B(1,3)-1, B(1,4)- and B(1,6)-glucans were present at the same concentrations in hyphal tips both in the presence or absence of 2,4-DAPG, as revealed by immunolocalization experiments using the primary antibody (de Souza et al. 2003a, b).

The genes required for the biosynthesis of 2,4-DAPG by *P. fluorescens* Q2-87 have been cloned and the biosynthetic locus includes *phlA, phlC, phlB* and *phlD*. A rapid polymerase chain reaction (PCR)-based assay targeting the *phlD* gene essential in phloroglucinol biosynthetic pathway was developed. The limited distribution of *phlD* among microbes makes it an ideal marker gene for 2,4-DAPG-producing *Pseudomonas* spp. present in the soil and other environmental samples to be detected (Fig. 5.1) (McSpadden Gardener et al. 2001). Likewise, the genes required for biosynthesis of phenazines, pyrrolnitrin and pyoluteorin have also been cloned and sequenced from different *Pseudomonas* spp. (Kirner et al. 1998; Mavrodi et al. 1998; Nowak-Thompson et al. 1999). Strains of *Pseudomonas fluorescens* were

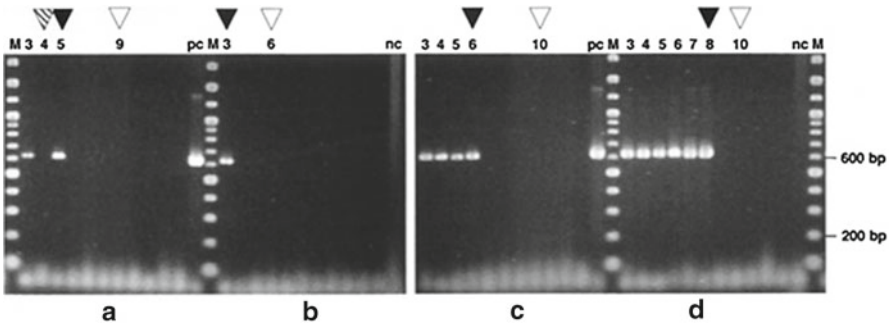

Fig. 5.1 Detection of *phlD* sequences in *Pseudomonas* spp. in the rhizosphere samples, grown in *Pseudomonas*-selective media using whole cell template for PCR amplification at different dilutions (*A, B, C* and *D*). Presence of *phlD*$^+$ bacteria in the serial dilution culture is indicated by 629- bp DNA product. *Lane M*: 100-bp DNA size standard; *numbers at the top* of the figure indicate the dilutions in the series (Courtesy of McSpadden Gardener et al. 2001 and with kind permission of The American Phytopathological Society, MN, USA)

screened for the production of 2,4-DAPG by employing a PCR-based screening procedure that used primers Phl2a and Phl2b. A fragment of 745-bp characteristic of the biosynthetic genes was amplified. The results of HPLC, HNMR and IR analysis also confirmed the ability of the strains to produce 2,4-DAPG. The purified antibiotic 2,4-DAPG was able to suppress the development of the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* in in vitro assays. This antibiotic preparation suppressed the development of bacterial blight disease up to 59–64 % in greenhouse and also in field evaluation (Velusamy et al. 2006).

The isolates (>300) of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. isolated from soils suppressive to Fusarium wilt or take-all disease were analyzed by whole-cell repetitive sequence-based PCR (rep-PCR) with the BOXAIR primers. The isolates belonging to seven genotypes A, D, E, L, O, P and Q were indentified. Fourteen isolates representing the different genotypes were evaluated for their ability to colonize the rhizosphere of pea plants. Population densities of genotypes D and P significantly increased faster and remained above 10^6 CFU/g of roots over the experimental duration of 15 weeks. The genetic profiles generated by the rep-PCR or RFLP analysis of the gene *phlD* indicated the rhizosphere competence of the 2,4-DAPG-prdoucing strains introduced into the rhizosphere (Landa et al. 2002). Strains of *Pseudomonas fluorescens* produce 2,4-DAPG which has a broad-spectrum of activity against many fungal plant pathogens as well as against the multidrug-resistant human pathogen *Staphylococcus aureus,* due to its bacteriolytic activity (Kamei and Isnansetyo 2003). However, the sensitivity of fungal pathogens *Pythium* spp. to 2,4-DAPG has been shown to vary significantly (Notz et al. 2001). In a later study, the sensitivity of 76 plant-pathogenic and/saprophytic strains of *Fusarium oxysporum* to 2,4-DAPG produced by *P. fluorescens* was assessed. *F. oxysporum* strains (17 %) including *F. oxysporum* f.sp. *melonis* (strain Fom38 and Fom1127) and *F. oxysporum* f.sp. *cubense* (strains Focub 1, 2 and 13) were relatively tolerant

to high concentrations of 2,4-DAPG. Some tolerant strains (18) were able to metabolize 2,4-DAPG. In two tolerant strains, deacetylation of 2,4-DAPG to less fungitoxic derivatives monoacetylphloroglucinol and phloroglucinol occurred. Fusaric acid produced by *F. oxysporum* strain might directly affect 2,4-DAPG biosynthesis by repressing the expression of the biosynthetic gene *phlA*. It was postulated that the presence of sublethal concentration of 2,4-DAPG may trigger fusaric acid production in *F. oxysporum* strains to repress 2,4-DAPG synthesis in *P. fluorescens* to prevent further accumulation of 2,4-DAPG in their habitat to toxic concentrations. Fusaric acid-mediated repression of 2,4-DAPG synthesis in *Pseudomonas* spp. was found to be strain-dependent, since fusaric acid blocked the 2,4-DAPG biosynthesis in strain CHA0, but not in strain Q21-87. The results suggested the need for the consideration of the fact that plant pathogens are dynamic organisms capable of rapidly adapting to and coping with adverse conditions like the presence of antagonistic microorganisms in the same habitat (Duffy et al. 2004; Schouten et al. 2004).

The 2,4-diacetylphloroglucinol (2,4-DAPG)-producing *Pseudomonas* spp. have been isolated from the rhizospheres of different crops grown in soils from diverse geographic regions (Keel et al. 1996). *P. fluorescens* UP61 was found to be effective against soilborne pathogens *Sclerotium rolfsii* infecting beans and *Rhizoctonia solani* infecting tomato. This strain produced three antibiotics viz., 2,4-DAPG, pyrrolnitrin and pyoluteorin contributing to its biocontrol activity. Molecular techniques such as 16S rDNA, RFLP, RAPD and rep-PCR assays and partial sequencing of the *phlD* gene governing the biosynthesis of 2,4-DAPG revealed the similarity of the strain UP61 with other biocontrol agents isolated from other geographical locations that have been shown to produce these antibiotics (De La Fuente et al. 2004). It is desirable to estimate the genotypic diversity of the 2,4-DAPG-producing *Pseudomonas* spp. that offers an enormous resource for identifying strains that are highly rhizosphere-competent and superior for biological control of crop diseases. A simple and rapid method was developed to detect the presence and assess the genotypic diversity of *ph1D*$^{+}$*Pseudomonas* strains directly in the rhizosphere samples without the need for prior isolation or enrichment on nutrient media. Denaturing gradient gel electrophoresis (DGGE) of 350-bp fragments of *phlD*$^{+}$ allowed discrimination between genotypically different *phlD*$^{+}$ reference strains and indigenous isolates. The DGGE analysis of the *phlD* gene allowed identification of new genotypic groups of specific antibiotic-producing *Pseudomonas* with different abilities to colonize the rhizosphere of sugarbeet seedlings (Bergsma-Vlami et al. 2005).

Pseudomonas fluorescens CHA0 produces antifungal antibiotics 2,4-DAPG and pyoluteorin (PLT) effective against several fungal pathogens and it promotes growth of plants also. Plant growth promotion may be due to solubilization of inorganic phosphates by production of organic acids especially gluconic acid which is one of the important acids produced by *Pseudomonas* spp. The role of gluconic acid produced by the strain CHA0 in phosphate solubilization was studied by producing mutants deficient in the genes encoding glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gad*) required for conversion of glucose to gluconic acid and 2-ketogluconate respectively. The ability of strain CHA0 to acidify the environment and to solubilize mineral phosphate was found to be strongly dependent on its

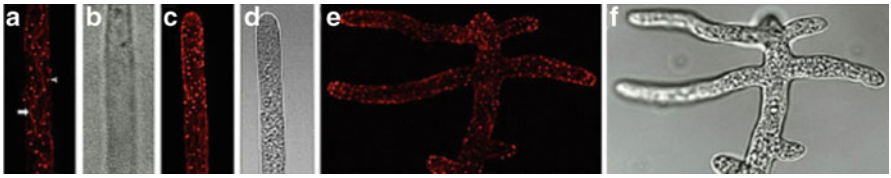

Fig. 5.2 Differential interference contrast (DIC) micrographs (**b**, **d** and **f**) and their corresponding confocal images (**a**, **c** and **e**) showing F-actin organization in control (**a**–**d**) and excessively branched and curled hyphae of *Aphanomyces cochlioides* by *Pseudomonas fluorescens* ECO-001; (**a**) and (**c**): normal organization of F-actin network; (**e**): disruption of F-actin in a *P. fluorescens*-hyperbranched hyphae; (**d**): DIC micrograph corresponding to (**c**); and (**f**): DIC micrograph corresponding to (**e**) (Courtesy of Islam and Fukushi 2010 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

ability to produce gluconic acid. The results indicated that formation of gluconic acid by CHA0 inhibited the production of PLT entirely, while production of DAPG was partially inhibited. In contrast, production of antifungal compounds by the mutant deficient in *gcd* was enhanced resulting in improved biocontrol activity against wheat take-all disease caused by *Gaeumannomyces graminis* var. *tritici.* The mutant was unable to produce gluconic acid. The study provided evidence to link the production of gluconic acid and loss or partial inhibition of ability to produce DAPG and PLT by the strain CHA0 (de Werra et al. 2009).

Pseudomonas fluorescens strain ECO-001 isolated from *Plantago asiatica* inhibited the polar growth of *Aphanomyces cochlioides,* causing damping-off disease of beet root and spinach on potato dextrose agar (PDA) medium by inducing excessive branching and curling of the hyphae. The antibiotic 2,4-DAPG secreted from the strain ECO-001 was found to be responsible for the observed abnormalities of the hyphae of *A. cochlioides.* The mechanism of action of 2,4-DAPG was not clearly understood, although its inhibitory effect on fungal pathogens has been widely reported. Tips of growing hyphae of Peronosporomycetes (earlier known as Oomycetes) contain an apical cap of the fine F-actin filaments adjacent to the plasma membrane, extending back along the hyphae and merge with subapical pattern of cortical actin cables and plaques (Bachewich and Heath 1998). To elucidate the mechanism of growth inhibition by ECO-001, a technique was developed to detect localized changes in the cortical filamentous actin (F-actin) organization by rhodamaine-conjugated phallodin (RP). Confocal laser scanning microscopic (CLSM) observations revealed that both ECO-001 and synthetic DAPG severely disrupted the organization of F-actin in *A. cochlioides* hyphae in a similar manner (Fig. 5.2). A known inhibitor of F-actin polymerization, latrunculin B also caused similar growth inhibition, excessive branching and induced disruption of F-actin in the pathogen hyphae. This investigation suggested that DAPG produced by ECO-001 might have direct effect on growth and development of *A. cochlioides* through disruption of cytoskeletal F-actin network. This report appears to be the first to indicate that disruption of cytoskeleton of a eukaryotic *A. cochlioides* by the metabolite DAPG secreted from a bacterial BCA (Islam and Fukushi 2010) (Appendix 5.1).

Pseudomonas fluorescens Pf-5 secretes a suite of secondary metabolites which are toxic to plant pathogens causing seed- and root rot diseases in several crops. The strain Pf-5 produces an array of toxic metabolites including pyoluteorin, pyrrolnitrin, 2,4-DAPG and hydrogen cyanide. Pyoluteorin and 2,4-DAPG are synthesized by polyketide synthase complexes and secreted from cells of Pf-5. Pyoluteorin production is governed by a biosynthetic gene cluster comprising of nine structural genes whose predicted functions entail the biochemical transformations required for pyoluteorin biosynthesis from acetate and proline precursors (Nowak-Thompson et al. 1999). Evidences strongly indicated that pyoluteorin served as an autoregulator, positively influencing its own production in *P. fluorescens* Pf-5. Exogenous application of pyoluteorin enhanced pyoluteorin production and exogenous pyoluteorin enhanced transcriptional activity of three pyoluteorin biosynthetic genes. Further, coinoculation with pyoluteorin-producing cells induced expression of pyoluteorin biosynthetic gene. In this respect, pyoluteorin functioned similar to established signaling molecules. Exogenous application of pyoluteorin repressed 2,4-dicetylphloroglucinol (2,4-DAPG) production by the strain Pf-5, as in the related strain *P. fluorescens* CHA0. Pyoluteorin and 2,4-DAPG mutually inhibited one another's production in Pf-5. The operation of pyoluteorin autoregulation in the rhizosphere was detected on cucumber seedlings in pasteurized soil with cross-feeding experiments. In the rhizosphere, expression of a pyoluteorin biosynthetic gene by a pyoluteorin-deficient mutant of Pf-5 was enhanced by pyoluteorin produced by coinoculated cells of Pf-5. This study showed that pyoluteorin has the ability to function as an intercellular signal between distinct populations of bacterial cells coinhabiting the rhizosphere. This finding appears to be the first to establish that pyoluteorin, an antibiotic could function as a single molecule in a natural environment (Brodhagen et al. 2004).

The communication mechanism between antagonistic *Pseudomonas* spp. and between rhizosphere bacterial communities has been studied to gain insight into the interactions between microorganisms that have a bearing on disease suppression and plant growth. Quorum sensing, the regulation of gene expression in response to the intracellular concentration of N-acylhomoserine lactones (AHLs), is a highly conserved mechanism utilized by a diverse range of Gram-negative bacteria (Narayanasamy 2008). The performance of *Pseudomonas* biocontrol agents is likely to be improved by applying mixtures of strains which are complementary in their capacity to suppress plant diseases. A combination of *P. fluorescens* CHA0 with *P. fluorescens* Q2-87 was studied in respect of the effect of these strain on each other's expression of a biocontrol trait. In both strains, production of 2,4-DAPG is a crucial factor contributing to the suppression of root diseases. DAPG acts as signaling compound, inducing the expression of its own biosynthetic genes. Dual cultural assays in which the two strains were grown in liquid medium physically separated by a membrane revealed that Q2-87 strongly induced *phlA* expression in a Δ*phlA* mutant of strain CHA0. Likewise, *phlA* expression on a Q2-87 background was induced by DAPG produced by CHA0. When coinoculated onto the roots of wheat seedlings grown under gonotobiotic conditions, the strains Q2-87 and CHA0, but not their respective DAPG-negative mutants, were able to enhance *phlA* expression

in each other. The results implicated that the positive cross-talk between two nonrelated pseudomonads might stimulate each other in the expression of an antimicrobial compound necessary for biocontrol activity (Maurhofer et al. 2004). In contrast, negative cross-communication among strains of *Pseudomonas aureofaciens* 30–84 in respect of expression of phenazine gene was observed. The presence of negative signal producing strains in a mixture with strain 30–84 reduced the ability of strain 30–84 to suppress the take-all disease pathogen *Gaeumannomyces graminis* var. *tritici* in vitro. The results suggested that cross-communication among members of the rhizosphere community and strain 30–84 might control secondary metabolite production and pathogen suppression (Morello et al. 2004).

A novel approach was adopted to monitor, in the rhizosphere, the expression of genes substantially contributing to the biocontrol efficacy of fluorescent pseudomonads. The biocontrol potential of *P. fluorescens* CHA0 is primarily determined by the production of antifungal compounds like 2,4-DAPG. The expression of the genes governing the synthesis of these compounds depended on abiotic and biotic environmental factors such as elements present in the rhizosphere. A new method for the in situ analysis of antifungal gene expression was employed, involving flow cytometry combined with green fluorescent protein (GFP)-based reporter fusions to the *phlA* and *prnA* genes essential for production of 2,4-DAPG and pyrrolnitrin respectively in strain CHA0. Expression of *phlA-gfp* and *prnA-gfp* in CHA0 cells harvested from the rhizosphere of a set of plant species, as well as from the roots of healthy, leaf pathogen-attacked and physically-stressed plants were analyzed. Levels of *phlA* and *prnA* expression varied significantly in the rhizosphere of different plant species. Physical stress and leaf pathogen infection lowered *phlA* expression levels in the rhizosphere of cucumber plants (de Werra et al. 2008).

Pseudomonas fluorescens strains CHA0 and Pf-5 produce the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin (PLT) with broad-spectrum antifungal and antibacterial activity (Haas and Keel 2003). The DAPG and PLT biosynthetic genes are located in the DAPG locus *phlABCD* and PLT locus *pltLABCDEFG* respectively. The role of the sigma factor RpoN (δ^{54}) in the regulation of the antibiotic production and biocontrol activity in *P. fluorescens* CHA0 was investigated. The *rpoN* mutant was defective for flagella and displayed drastically reduced in swimming and swarming motilities. However, the *rpoN* mutant produced DAPG in greater concentrations (several folds) and showed higher level of expression of the biosynthetic gene *phlA,* compared with the wild-type strain and the mutant complemented with a single copy of $rpoN^+$. In contrast, loss of RpoN function led to marked reduction in PLT production and *plt* gene expression, suggesting that RpoN may control the balance of the antibiotics DAPG and PLT in the strain CHA0. In the natural soil, the *rpoN* mutant was found to be less efficient in suppressing the root rot disease in cucumber caused by *Pythium ultimum*, although the mutant was not significantly impaired in its root colonization capacity even at early stages of root infection by *Pythium* spp. (Table 5.2). The results confirmed that RpoN was the major regulator of the biocontrol activity in *P. fluorescens* CHA0 (Péchy-Tarr et al. 2005).

Table 5.2 Impact of R_{poN} on the suppression of *Pythium* damping-off and root rot of cucumber by Pseudomonas fluorescens CHA0 in natural soil (Péchy-Tarr et al. 2005)

Bacterial strain tested	*Pythium* added	Surviving plants/ flask (%)	Colonization of roots by *P. fluorescens* (Log_{10} CFU/g of roots)
None	–	100a	ND
CHA0 C wild type	–	99a	6.67 ± 0.37
CHA 250 (ΔrpoN)	–	100a	6.55 ± 0.15
CHA 251 (ΔrpoN alt Tn 7 :: $rpoN^+$)	–	98ab	7.19 ± 0.24
CHA0/pME 8013 (PrpoN – $rpoN^+$)	–	97ab	6.62 ± 0.10
CHAO/ pME 8014 ($Plac^-$ $rpoN^+$)	–	100a	6.53 ± 0.02
None	+	63c	ND
CHA0	+	92ab	7.56 ± 0.43
CHA 250	+	73c	7.20 ± 0.73
CHA 251	+	90b	7.30 ± 0.15
CHA0/pME8013	+	95ab	7.44 ± 0.16
CHA0/pME3014	+	93ab	7.57 ± 0.48

x – Data represent the means from six individual repetitions; means followed by the same letter in a column are not statistically different (P = 0.05) as per Fisher's least significant difference
ND not detected

Pseudomonas fluorescens CHA0 produces two potent antibiotics 2,4-DAPG and pyoluteorin (PLT) which have been shown to be primarily responsible for its biocontrol activity against several plant pathogens. Regulation of DAPG and PLT biosynthesis is complex and consists of several levels of pathway-specific and global control. DAPG strongly represses PLT biosynthesis and vice-versa, pointing to a mechanism of mutual feed back control that might help *P. fluorescens* to keep production of these compounds at balanced levels (Baehler et al. 2005). The exoproducts of the strain CHA0, the two proteins MVaT and MvaV were characterized and their potential as novel regulators of the biocontrol activity of the strain CHA0. In *mvaT* and *mvaV* in frame-deletion mutants of strain CHA0, PLT production was enhanced by about 4-and 1.5-fold respectively, whereas DAPG production remained as in wild-type strain. On the other hand, in a double mutant with disrupted *mvaT* and *mvaV*, PLT production was enhanced up to 20-fold, while DAPG biosynthesis was entirely repressed. The effects of mutations on antibiotic biosynthesis were confirmed by following expression of *gfp*-based reporter fusions to the corresponding biosynthetic genes. Biocontrol activity was almost entirely abolished in double mutant. The results indicated that MvaT and MvaV could act together, as further regulatory elements in the complex network, controlling expression of biocontrol traits of *Pseudomonas* spp. (Baehler et al. 2005).

Pseudomonas fluorescens strain KD does not produce 2,4-diacetylphloroglucinol (2,4-DAPG) which is the principal antibiotic involved in the biocontrol activity of several strains of *Pseudomonas* spp. However the strain effectively protected

cucumber plants against the damping-off disease caused by *Pythium ultimum*. The type III secretion system (TTSS) is employed by bacteria for pathogenic or symbiotic interaction with plant and animal hosts. The presence of TTSS genes in *P. fluorescens* KD was detected. Despite the presence of pathogenic attribute, the strain did not behave as a phytopathogen. Inactivation of *hrcV* strongly reduced the biocontrol potential of the strain KD against damping-off disease in cucumber. The reduced biocontrol efficacy was not due to a lower ecological fitness of *hrcV* mutant, because the mutant persisted in the potting mix and colonized the plant roots to the same level as that of the wild-type strain, regardless of whether the pathogen was present or not. The expression of the operon containing *hrcV* in the strain KD was strongly stimulated in vitro and in situ by *P. ultimum,* but not by cucumber (Rezzonico et al. 2005).

Phenazines

The biocontrol efficacy of *Pseudomonas chlororaphis* PCL 1391, *P. aeruginosa* PNA1 and 7NSK2 and *Pseudomonas* CMR12a in suppressing the development of *Verticillium longisporum,* infecting cauliflower, was assessed using a microplate assay. *P. chlororaphis* PCL 1391 was the most effective in suppressing the microsclerotia of *Verticillium* by the activity of phenazine-1-carboxamide (PCN) (Appendix 5.2). A seven-gene operon phzABCDEFG is responsible for the synthesis of phenazine-1-carboxylic acid (PCA) and *phzH* encodes for an asparagine synthetase like enzyme that is responsible for the conversion of PCA to PCN. The involvement of PCN in *P. chlororaphis* PCL 1391-mediated reduction of microsclerotia viability was studied by using the mutants deficient in *phzB* and *phzH* genes. A *phz B* mutant (PCL 1119) was phenzine-deficient and a *phzH* mutant (PCL1121) produced PCA instead of PCN. PCL 1119 was as effective in inhibiting microsclerotia germination as the wild-type, whereas PCL 1121 was more effective, when compared to the wild-type. In addition, involvement of PCA in the suppression of germination of microsclerotia could be demonstrated. The mutants of *P. chlororaphis* PCL 1391 and *P. aeruginosa* 7NSK2 overproducing PCA were more effective in inhibiting microsclerotia germination and formation of secondary microsclerotia, when compared to wild-type strain. The strains of *Pseudomonas* produced biosurfactants that might facilitate adhesion of the bacteria on the surface of fungal pathogens and act synergistically with the antibiotics to increase the effectiveness of biocontrol by the bacterial BCA (Debode et al. 2007).

Pseudomonas chlororaphis strain PCL 1391 produces phenazine-1-carboxamide (PCN) crucial for its biocontrol activity against tomato wilt pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). In addition, the strain PCL 1391 produces hydrogen cyanide (HCN), chitinase and protease. The expression of the biosynthetic operon for PCN was shown to be under regulation of quorum sensing (Chin-A-Woeng et al. 2001a, b). Additional genes contributing to the regulation of phenazine biosynthesis were identified. Screening for PCL 1391 transposon mutants for increased PCN production resulted in the identification of the *psrA* gene. The transcriptional activity of *psrA* was profiled along with the quorum-sensing regulatory genes and

phz biosynthetic genes during growth. In addition to the already identified *phzI/phzR* and *gac* regulatory genes, the *psrA* gene also significantly contributed to the regulation of PCN biosynthesis. In addition, the repressing effect of *psrA* expression on quorum-sensing genes, *phz* biosynthetic genes and on itself was also demonstrated in this study by Chin-A-Woeng et al. (2005). *Pseudomonas chlororaphis* GP72 isolated from the rhizosphere of green pepper plants produced the phenazines mainly phenazine-1-carboxylic acid (PCA) and 2-hydroxy-phenazine (2.0H-PHZ) which showed broad spectrum of antifungal activity against plant pathogen (Liu et al. 2007). The alternative sigma factor RpoN functions in concert with specialized transcriptional activators (enhancer-binding proteins) to control the expression of genes coding for very diverse functions in response to environmental stimuli. In pseudomonads the features that depend on RpoN include motility and diverse metabolic functions, such as transport and metabolism of various nitrogen and carbon sources (Köhler et al. 1989). The effect of *rpoN* gene chromosomal inactivation on production of antifungal compound was assessed by inoculating the parental strain GP72 and the *rpoN* mutant GP72N were inoculated in King's Medium B (KMB) and Pigment Producing Medium (PPM). The parental strain produced significantly higher quantities of PCA than the mutant in both media tested. Complementation of the *rpoN* gene in mutant GP27N restored the ability to synthesize PCA as effectively as the parental strain and its motility. The results showed that PCA biosynthesis was partially repressed by the deletion of RpoN in *P. chlororaphis* GP 72 strain. The results suggested that RpoN might be involved as a positive regulator in the regulation of PCA biosynthesis in *P. chlororaphis* GP 72 (Liu et al. 2008a, b).

The putative role of phenazines produced by *Pseudomonas aeruginosa* PNA1 effective against *Pythium splendens* (infecting bean) and *P. myriotylum* (infecting cocoyam) was investigated. The biological activity of PNA1 was attributed to the production of phenazine-1-carboxylate (PCA) and phenazine-1-carboxamide (PCN), since its tryptophan autotrophic mutants FM13 deficient in phenazine production was unable to protect the plants against the oomycete pathogens. Exogenous supply of tryptophan restored the biocontrol activity of FM13 strain, as reflected by the reduction in disease severity in cocoyam plants. (Tambong and Höfte 2001). Two *Pseudomonas* strain isolated from the rhizosphere of cocoyam plants exhibited excellent biocontrol activity in vivo against *Pythium myriotylum* to a level similar to that of *P. aeruginosa* PNA1. They were designated *Pseudomonas* CMR5C and CMR12a. These strains produced phenazines and surfactants. Strain CMR5C formed pyrrolnitrin and pyoluteorin also (Perneel et al. 2007). In a later study, the phenazine mutant FM29 and rhamnolipid (surfactant) mutant PNA1-Rhl of *P. aeruginosa* PNA1 applied separately or with sterile volcanic soil, no suppressive effects were observed. The wild-type strain PNA1 significantly suppressed root rot disease, compared with the mutant strains. When both mutants were applied concurrently, the biocontrol efficacy exceeded that of parental strain PNA1. Bean seeds developed less reduction in pre-emergence damping-off caused by *P. splendens,* when treated with a mixture of purified PCN and rhammolipids. Microscopic observations revealed substantial vacuolation and disintegration of hyphal cells of *Pythium,* after incubation with liquid medium amended with both PCN and rhamnolipids (Perneel et al. 2008).

Interactions between two bacterial biocontrol strains of *Pseudomonas chlororaphis* PCL 1391 and *P. fluorescens* WCS 365 with *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), causing tomato foot and root rot disease, were visualized in confocal laser scanning microscope (CLSM), using different autofluorescent proteins as markers. Tomato seedlings were bacterized with the strains PCL1391 and WCS 365 and planted in sand system infested with FORL. The bacterial strains reached root surface earlier and multiplied faster than the pathogen. The bacteria and fungal hyphae colonize the same niches on the tomato root, namely, the intercellular junctions. This might be due to chemotaxis toward and utilization of exudates compounds. By colonizing these sites and utilizing the exudates nutrients, the bacteria prevent colonization and penetration of the root tissue by FORL. The strain PCL 1391 produced phenazine-1-carboxamide (PCN) which altered the growth and morphology of fungal hyphae both in vitro and in vivo (greenhouse condition). The lack of PCN production in the strain PCL119 resulted in a delay in the appearance of morphological alterations of hyphae. In the case of the strain WCS 365, induced systemic resistance (ISR) is considered to play a major role in the mechanism of biocontrol activity of this strain. However, no differences between the effects on the FORL by WCS 365 and PCL1391 that could be related to ISR, were observed. Probably the effects of ISR by WCS 365 were more or less compensated for by the antibiosis effect of the strain PCL 1391. The extensive root colonization by both bacterial strains may represent a new mechanism in biocontrol by these *Pseudomonas* strains (Bolwerk et al. 2003).

Pseudomonas chlororaphis strain PCL 1391 and *P. fluorescens* WCS 365 were evaluated for their efficacy in suppressing the development of three races of *Colletotrichum lindemuthianum* in bean plants. Disease development was suppressed most effectively by the strain PCL 1391, whereas the strain WCS 365 exhibited no significant difference compared to the positive control. Combination of the two strains was no better than *P. chlororaphis* alone. Both strains proved to be excellent colonizers of bean roots and their combined treatment resulted in increased total bacterial populations on the root tips. Yet the population of *P. fluorescens* was reduced in the presence of *P. chlororaphis. P. chlororaphis* adversely affected the growth, sporulation and conidial germination of all the three races of *C. lindemuthianum*. In contrast, there was no effect on these characteristics of the pathogen races following treatment with *P. fluorescens*. The antibiotic effect of *P. chlororaphis* on the pathogen was considered to be due to its ability to produce phenazine-1-carboxamide which was detected using thin layer chromatography (TLC) technique. The results indicated the effectiveness of *P. chlororaphis* in protecting the bean plants against the races of anthracnose pathogen tested (Bardas et al. 2009). *Pseudomonas chlororaphis* strain PA 23 provided effective protection to canola and sunflower against *Sclerotinia sclerotiorum.* The strain PA23 produced the non-volatile antibiotics phenazine and pyrrolnitrin as well as the volatile antibiotics nonanal, benzothiazole and 2-ethyl-1-hexanol. In the greenhouse, the role of the nonvolatile antibiotics on root colonization and biocontrol ability of PA23 against *S. sclerotiorum* on sunflower was studied. Application of the strain PA23 alone or in combination with phenazine- and pyrrolnitrin-deficient *Tn* mutants resulted in significantly higher

(P = 0.05) root bacterial number and suppression of Sclerotinia wilt disease. The bacterial population decreased considerably and it appeared to be negatively correlated with the number of antibiotics produced by PA23. The role of phenazine or pyrrolnitrin was not clearly discernible from the results. The strains producing at least one antibiotic were able to maintain relatively higher population than non-producers. However, an increase in bacterial number increased at 6 weeks after sowing, in the case of strains producing at least one antibiotic. Combination of PA23 with certain mutants led to reduced root colonization and biocontrol potential of the strain PA23 (Athukorala et al. 2010).

The phenazine biosynthetic locus consists of seven genes (*phzABCDEFG*) arranged in a single operon in *Pseudomonas chlororaphis*. A pLAFRI cosmid clone (p06phz) which carried the biosynthetic genes of *P. chlororaphis* 06 was introduced into *Rhizobium etli* USDA 9032 by triparental mating with pRK2013 as helper plasmid. Thin-layer chromatography analysis with Silica Gel G chromatography showed that *R. etli* carrying the *Phz* genes produced a yellow-colored compound that had an Rf value similar to the hydroxyl phenazine-1-carboxylic acid. *P. chlororaphis* 06 produced two more compounds with Rf values similar to those for 2-phenazine carboxylic acid and 2-hydroxyl phenazine which were not detected in the *R. etli* transconjugants. The phenazine-producing *R. etli* transconjugants could inhibit the mycelial growth of *Botrytis cinerea* and *Fusarium oxysporum* in the plate assay like *P. chlororaphis* 06. In contrast, *R. etli* carrying the cloning vector alone did not inhibit the pathogen growth. The consequences of phenazine production on the symbiotic performance of the phenazine-producing *R. etli* were assessed. The transconjugant grew poorly on YEM broth and the phenazine production inhibited the viability of the cells. Black bean inoculated with phenazine-producing strain produced pale green leaves indicative of nitrogen deficiency. Numerous brownish small nodules produced, did not exhibit acetylene reduction activity, indicative nitrogen fixation. The engineered strain, although produced the antibiotic, lacked the capacity for nitrogen fixation and its growth was inhibited by phenazine, making the utility of the engineered strain very much limited as a biocontrol agent (Krishnan et al. 2007).

Bacterial isolates (105) obtained from rhizosphere soils, roots, stems and leaves of winter wheat, grown in irrigated and rainfed fields, exhibited antagonistic activity against *Gaeumannomyces graminis* var. *tritici* in vitro tests. These isolates were identified as *Pseudomonas* spp. by amplified ribosomal DNA restriction analysis. Based on biocontrol assays, 13 strains, were selected, because of their aggressive colonization of wheat rhizosphere and effective suppression of wheat take-all disease. Three of the thirteen strains, HC9-09, HC13-07 and JC 14–07 (all stem endophytes) had genes for the biosynthesis of phenazine-1-carboxylic acid (PCA), but none possessed genes for the production of 2,4-diacetylphloroglucinol, pyoluteorin or pyrrolnitrin. In addition, production of PCA by these strains was corroborated by high-pressure liquid chromatography (HPLC) analysis of 2-day old cultures. HPLC quantitative time-of-flight 2 mass-spectrophotometry analysis of extracts of roots of spring wheat colonized by HC9-07, HC13-07 or *P. fluorescens* 2–79 revealed that all three strains were able to produce PCA in the wheat rhizosphere.

Ability to produce PCA by strain HC 9–07 resulted in the loss of biocontrol activity indicating the initial role played by PCA in suppressing take-all disease development. Analysis of DNA sequences within the key phenazine biosynthesis gene *phzF* and of 16S rDNA indicated that strains HC9-07, HC 13–07 and JC 14–07 were similar to the well-established PCR producer *P. fluorescens* 2–79 (Yang et al. 2011).

Pseudomonas strain LBUM223 inhibited in vitro growth of several fungal pathogens such as *Botrytis cinerea*, *Phytophthora cactorum* and *Sclerotinia sclerotiorum* and it carried phenazine biosynthetic genes involved in the production of phenazine-1-carboxylic acid (PCA) (Paulin 2007). The ability of the strain LBUM 223 to suppress the potato common scab disease caused by *Streptomyces scabies* was assessed. The pathogen produced thaxtomins, involved in inducing disease symptoms on tubers. The involvement of PCA in repressing thaxtomin biosynthesis genes *txtA* and *txtC* and control of potato common scab disease was investigated, using a mutant deficient in PCA production (LBUM *223 phzC*$^{-}$). Seed piece tuber treatment with LBUM 223 before planting in pathogen-infested soil suppressed the disease development. A relationship was demonstrated between PCA-producing ability of the strain LBUM 223 and its capacity to inhibit the growth of *S. scabies* and its ability to repress the biosynthesis genes *txtA* and *txtC* in pathogen. The mutant LBUM 223 *phzC*$^{-}$ was less efficient in reducing the growth of *S. scabies* and it could not repress the thaxtomin biosynthetic genes. The results suggested that production of PCA by the strain LBUM 223 might have a key role in limiting pathogen development, repressing the expression of pathogenicity genes *txtA* and *txtC* and consequently in limiting the surface area of progeny tubers covered with common scab lesions (St-Onge et al. 2011). The antagonistic potential of *Pseudomonas* sp. LBUM300 in suppressing the development of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causing tomato bacterial canker disease was determined. *Pseudomonas* sp. LBUM300 produced both DAPG and HCN on *Cmm* under in vitro and in planta conditions. Nonsynthesizing isogenic mutants of the producer strains were generated to dissect the role of individual metabolite on *Cmm* biological control. Novel specific quantitative PCR TaqMan assays allowed quantification of *Cmm* in tomato plants and rhizospheric soil. *Pseudomonas* sp. LBUM223 and LBUM300 strains significantly repressed *Cmm* growth in vitro, while their respective non-producing mutants showed less or no significant antagonistic activity. In planta, only *Pseudomonas* sp. LBUM 300 could reduce disease development and *Cmm* rhizospheric population. The results suggested that production of both DAPG and HCN contributed to the antagonistic ability of *Pseudomonas* sp. LBUM 300 strain. Simultaneous production of DAPG and HCN by LBUM 300 makes it a desirable candidate for further advancement in commercialization for the biological control of tomato bacterial canker disease (Lanteigne et al. 2012).

Pseudomonas CMR12a was effective against *Rhizoctonia solani* causing root rot disease of bean and it produced phenazines and cyclic lipopeptides (CLPs). The involvement of phenazines and CLPs in the biocontrol activity of the strain CMR 12a was investigated, using two different anastomsis groups AG2-2 (intermediately aggressive) and AG4HGI (highly aggressive). The wild-type strain CMR12a reduced drastically the disease severity caused by both *R. solani* AGs.

A CLP-deficient and a phenazine-deficient mutant of CMR12a could still protect the bean plants with less efficiency, compared with wild-type strain. The biocontrol activity was lost entirely, when the mutant was deficient in production of both antibiotics. Washing of bacterial cells before application resulted in significant reduction in disease suppressive ability of the wild-type strain, indicating that removal of metabolites produced during growth on plate was necessary for retaining higher level of biocontrol efficacy. The observations under the microscope revealed pronounced branching of hyphal tips of both *R. solani* AGs in the presence of CMR12a strain. Phenazine-deficient mutant induced more branched and denser mycelium, whereas CLP-deficient mutant and mutants deficient in both CLPs and phenazine did not alter the vegetative growth of the pathogen. The results indicated that phenazine and CLPs might have role in the suppression of development of Rhizoctonia root rot disease of bean (D'aes et al. 2011). In *Pseudomonas aeruginosa* PAO1, two genes *PhzM* and *PhzS* were characterized. They code for enzymes that modify phenazine into its related derivatives. The gene *phzM* is located upstream of *phZA1B1C1D1E1F1G1* operon and it is involved in the production of pyocyanin (Mavrodi et al. 2001). The purified pyocyanin from *P. aeruginosa* TO3 was evaluated for its antifungal activity against *Macrophomina phaseolina.* Purified pyocyanin inhibited the growth of the pathogen. Using a well-diffusion method, the effect pyocyanin on disease suppression and biofilm formation by the rhizobial strain Cal2 on radicles of groundnut (peanut) was assessed. Pyocyanin suppressed disease more effectively at high concentration. However, at lower concentration pyocyanin, increased colony-forming units of Ca12 on radicles of seedlings were observed. Application of pyocyanin-producing pseduomonads together with rhizobia contributed to the enhancement of modulation ability and sustained the growth and productivity of groundnut even in the presence of the destructive pathogen *M. phaseolina* (Khare and Arora 2011).

Rhizospheric bacterial antagonists isolated from cabbage of rhizosphere were evaluated for their efficacy in suppressing the development of blackrot disease caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*). Of the three strains, TO7 was the most effective in suppressing the disease symptoms than the other two strains SA3 and CA9. The strain TO7 was tested in the field and it was very effective in controlling black rot disease in cabbage, when applied through root dip method. Based on the nucleotide homology and phylogenetic analysis, TO7 was identified as nearest homolog of *Pseudomonas fluorescens*. The strain T07 produced 2,4-diacetylphloroglucinol (2,4-DAPG) which was analyzed in different physiological conditions through qRT-PCR analysis of *phlD* gene, followed by its subsequent effect on antagonistic activity. The TO7 culture of mid log and extended stationary growth phase with shaking had a maximum *phlD* expression. Growth temperature and pH exerted direct effect on *phlD* expression with maximum production at 16 °C and at alkaline pH 8.5. The results showed that *P. fluorescens* strain T07 had potential for effective suppression of the black rot disease of cabbage and its mode of action was through production of the antibiotic 2,4-DAPG (Mishra and Arora 2012).

Pyrrolnitrin

Many fluorescent and non-fluorescent *Pseudomonas* spp. have been reported to produce the antibiotic pyrrolnitrin (PRN). *P. fluorescens, P. chlororaphis* and *P. aureofaciens* are able to secrete PRN antibiotics that are effective against fungal pathogens such as *Botrytis cinerea* (Hammer and Evensen 1993) and *Gaeumannomyces graminis* var. *tritici* (Tazawa et al. 2000) and *Sclerotinia sclerotiorum* (Fernando et al. 2005). Intensification of research on the biocontrol potential of various microorganisms belonging to diverse classes has been made to find alternative methods for managing microbial plant pathogens which developed resistance to currently applied fungicides or other chemicals. However, it may be possible to have similar loss of efficacy of biological control agents. For example, about 17 % of strains of *Fusarium oxysporum* were found to be naturally tolerant to the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) produced by *Pseudomonas* spp. Likewise, some isolates of *Botrytis cinerea* could also tolerate 2,4-DAPG (Schouten et al. 2004, 2008).

In order to assess the sensitivity of *Botrytis cinerea* to pyrrolnitrin, the baseline sensitivity distribution of *B. cinerea* populations to pyrrolnitrin-producing strain PhZ24 of *Pseudomonas chlororaphis* was assessed in vitro. The efficacy of the strain PhZ24 was assessed in in vitro confrontation assay on TSA medium containing Tryptic Soy broth (3 g) and agar (15 g) against 12 selected isolates of *B. cinerea*. The inhibition zones were measured (in mm) from 5 days after inoculation. The efficacy of the strain PhZ24 against *B. cinerea* isolates on tomato plants was determined. The EC_{50} values of 166 isolates of *B. cinerea* based on spore germination ranged from 0.0038 to 0.0318 μg/ml of pyrrolnitrin. The most resistant isolate was separated from the most sensitive isolate by a factor of 8.4 arrived at by dividing the EC_{50} value of the least sensitive isolate by the EC_{50} value of the most sensitive isolate. The wide variation of sensitivity suggested that *B. cinerea* might have the potential to evolve to some level of resistance to pyrrolnityrin. However, all isolates (12) tested against the strain PhZ24 displayed a similar level of sensitivity, whatever their EC_{50} values were in vitro. In addition, on tomato plants, protection level offered by the bacterial BCA was between 96 and 100 %, indicating high potential of the bacterial strain PhZ24. No significant differences in the responses could be recorded between the isolates of *B. cinerea*. This may be possibly, because *P. chlororaphis* PhZ24 is also able to produce another antibiotic phenazine-1-carboxylic acid (PCA) (Ajouz et al. 2009).

Botrytis cinerea, causative agent of gray mold diseases of tomato and a range of plant species, exhibits high genetic variability and it is considered a high-risk pathogen in terms of development of resistance to fungicides (Leroux 2004). Mutants of *B. cinerea* differing in their level of resistance to pyrrolnitrin [3-chloro-4 (2´-nitro-3´-chlorophenyl)-pyrrole] were selected. Development of resistance to pyrrolnitrin in four strains of *B. cinerea* was consistently associated with a dramatic loss of aggressiveness on tomato and apple fruits (Ajouz et al. 2010). The behavior of two near-isogenic lines of *B. cinerea* with differing level of resistance to pyrrolnitrin on artificially inoculated petioles and stems of tomato plants was compared by

using histopathological procedures. Differences in the aggressiveness between the pyrrolnitrin resistant mutant BCG20P and wild-type parent BC1GO were not related to the early stages of infection. The conidial germination, proliferation of germ tube and colonization of petioles were similar in the mutant and wild-type isolates. Both isolates produced mycelium within tomato petiole tissues, but the mutant generally failed to spread further to the stem. The absence of mycelium in the stem in the case of slow-growing mutant might be due to the limited induction of reactive oxygen intermediates involved in the HR reaction of the plant. Development of resistance to pyrrolnitrin in *B. cinerea* sounds a caution on the possibility of the pathogen developing resistance to the biocontrol agents that primarily suppress the development of pathogens by producing antibiotics (Ajouz et al. 2011a, b).

Pseudomonas fluorescens strain MM-B16 isolated from a mountain forest soils in Korea was identified based on the physiological and biochemical characteristics and 16S rDNA sequence analysis. The culture filtrates of strain MM-B16 were assayed for their antifungal properties, using the pathogen *Colletotrichum orbiculare* infecting cucumber, *Phytophthora capsici* and *Pythium ultimum* infecting pepper (chilli) in in vitro tests. The active compound present in the culture filtrates was identified as a thiazoline derivative, aerugine [4-hydroxy-methyl-2-(2-hydroxyphenyl)-2-thiazoline]. The antibiotic was effective against the pathogens of cucumber and pepper, but not against yeasts and bacteria. Aerugine was relatively more effective against *C. orbiculare* and *P. capsici,* compared with level of protection against *P. ultimum.* However, the efficacy of aerugine was less than the fungicides metalaxyl and chlorothalonil (Lee et al. 2003). *Botrytis cinerea* isolates (204) were tested to establish a baseline sensitivity to pyrrolnitrin antibiotic. The pathogen isolates exhibited a range of sensitivity to pyrrolnitrin with an 8.4-fold difference in EC_{50}, using reduction in spore germination by 50 % as the basis. The less sensitive isolates showed in sensitivity to other antibiotics also. The efficacy of the pyrrolnitrin-producing *Pseudomonas chlororaphis* ChPhzS24 strain was tested in vitro and on tomato plants by inoculating *B. cinerea* isolates with differing sensitivity to pyrrolnitrin determined on the EC_{50} values. Although the *B. cinerea* isolates had different EC_{50} values, no significant differences in the sensitivity of pathogen isolates could be observed to the ChPhzS24 strain. The results indicated that the pathogen isolates differed in their sensitivity to the purified antibiotic, but not to the BCA strain which could suppress the pathogen development through other mechanisms, in addition to the antibiotic produced by the BCA strain concerned (Ajouz et al. 2011a, b).

Strains of *Pseudomonas chlororaphis* PA23, *Pseudomonas* strain DF41 and *Bacillus amyloliquefaciens* BS6, capable of inhibiting infection of canola petals by *Sclerotinia sclerotiorum* in both greenhouse and field evaluation, *Bacillus thuringiensis* BS8, *B. cereus* L and *B. mycoides* S showing inhibitory effects in in vitro tests were examined for the presence of antibiotic-specific genes by applying PCR assay and Southern blotting. Thirty primers were employed to amplify antibiotic biosynthetic genes encoding phenazine-1-carboxylic acid (PCA). 2;4-DAPG, pyoluteorin and pyrrolnitrin and the zwittermicin A self-reliance gene. The PA sequence showed that it had >90 % identity with phenazine biosynthetic genes from three strains of

P. chlororaphis and it also showed 93 % similarity to the *phzCD* sequence from the positive control strain *P. fluorescens* 2–79. The PCR product (1,050-bp) amplified from *P. chlororaphis* PA23 by primers Prn AF/PrnAR exhibited high similarity (>90 %) to pyrrolnitrin biosynthetic genes *prnABCD* of *P. fluorescens* and *P. chlororaphis*. The presence of pyoluteorin and 2,4-DAPG biosynthetic genes could not be detected in *P. chlororaphis*, as there was no PCR-amplified product by employing specific primers. Production of phenazine and pyrrolnitrin by *P. chlororaphis* was confirmed by HPLC technique. *Pseudomonas* spp. DF41 and *B. amyloliquefaciens* BS6 lacked the genes for biosynthesis of any of the antibiotics tested. *B. thuringiensis* BS8, *B. cereus* L and *B. mycoides* possessed the zwittermicin A self-resistance gene (Zhang et al. 2006).

Pseudomonas chlororaphis strains DF 190 and PA23, *Bacillus cereus* strains DFE4 and *B. amyloliquefaciens* strains DFE16 were effective against canola blackleg disease caused by *Leptosphaeria maculans* (anamorph: *Phoma lingam*). These antibiotic-producing strains were evaluated for their ability to induce systemic resistance (ISR) by split-inoculation (SPI) of a cotyledon different from that of the pathogen inoculated cotyledon. Application of the bacteria 24 or 48 h prior to pathogen inoculation was an important factor in the suppression/prevention of blackleg lesions. The strains PA23 and DF190 produced phenazines (phenazine-1-carboxylic acid, 2 hydroxyphenazine) and pyrrolnitrin and the strains DFE4 and DFE16 produced the lipopeptide antibiotics iturin A, bacillomycin D and surfactin. The strains PA23 and DFE4 were tested as representative producers of each set of antibiotics for the split inoculation of the extracts for induction of ISR. The assays showed a small, but significant reduction in disease severity via a systemic response. The local (SPI) inoculation of the extract (for direct antagonism) showed significantly higher reduction of disease severity which was also consistent with the SPI of the bacterial cells, establishing a more important role for the antifungal metabolites present in the culture extracts for the direct suppression of the development of blackleg symptoms. The localized inhibition of pycnidiospores by the bacteria could be due to successful colonization of the infection site which in turn most possibly act as a suitable delivery system for the antifungal metabolites (Ramarathnam et al. 2011).

The GacS/GacA system in known to control the expression of genes required for the synthesis of secondary metabolites such as antibiotics in several *Pseudomonas* spp. *A gacS* mutant lost the biocontrol activity against *Leptosphaeria maculans*, causative agent of canola blackleg disease. The biocontrol activity could be restored in the mutant PA23-314, when the *gacS* gene was added to the mutant. The phenazine mutant PA23-63 showed antifungal and biocontrol activity similar to that of the wild-type strain (Selin et al. 2010). The biocontrol activity of the mutant PA23-63, though lacked phenazine production, revealed that phenazines were not required for the biocontrol of *L. maculans*. The results showed that localized plant-defense-related enzyme activity at the site of inoculation was not induced by the bacterial strains of *P. chlororaphis*. Direct antifungal activity at the site of infection appeared to be the dominant mechanism mediating control of blackleg disease of canola (Ramarathnam et al. 2011).

Production of antibiotics by bacterial biocontrol agents may become an unacceptable attribute for their use against postharvest pathogens affecting fruits and vegetables, although they protect the treated produce against the pathogens effectively. *Pseudomonas cepacia* strain LT-4-12-W produced pyrrolnitrin and also reduced the in vitro growth and conidial germination of *Monilinia fructicola,* causing peach brown rot and *Botrytis cinerea* causing gray mold disease of apple and pear (Pusey and Wilson 1984; Janisiewicz and Roitman 1988). However, the production of antibiotics as a critical factor in biocontrol potential of these BCAs is not established unequivocally, since *P. cepacia* could be employed for the control of green mold disease of lemon caused by a pyrrolnitrin-resistant strain of *P. digitatum* (Smilanick and Denis-Arrue 1992). Likewise, use of the strain LT-4-12-W of *P. cepaciae* resulted in significant control of blue mold decay on oranges inoculated with pyrrolnitrin-resistant mutants of *Penicillium italicum* (Janisiewicz and Korsten 2002).

Penicillium digitatum causing green mold and *P. italicum* inducing blue mold diseases of citrus could be controlled by the application of *Pseudomonas syringae* strains, ESC-10 and ESC-11, the principal ingredients of the commercial product BioSave. These strains produced syringomycin E. Although the purified syringomycin could inhibit the growth of a variety of fungi, the presence of syringomycin was not detected in the fruit wounds treated with *P. syringae*, raising doubt as to the role of antibiotic in disease control (Bull et al. 1997) and suggesting the operation of a different mechanism not dependent on the production of syringomycin. The *syrB* mutants of strains ESC-10 and ESC-11 were generated by disrupting the *syrB* biosynthesis gene by a *lacZ* reporter gene coding for β-galactosidase activity. In cultures inoculated with the *syrB* mutant of strain ESC-10, ß-glucosidase activity was higher in media containing albedo tissue after 4 days of incubation. The reporter-gene system has been used to determine the production of syringomycin E by these strains in wounds on lemons or oranges (Bull et al. 1998). However, it may not be feasible for the antibiotic-producing BCAs to be registered for postharvest use on food products, because of the concern relating to the introduction of antibiotics into human food which may have an adverse effect on the resistance of humans to antibiotics. In addition, inhibition of microbial pathogens by a single chemical compound may prove to be ineffective, when a strain of the pathogen resistant to the antibiotic develops.

Hydrogen cyanide (HCN) is a volatile antibiotic produced as a secondary metabolite by Gram-negative bacteria *P. fluorescens, P. aeruginosa* and *Chromobacterium violaceum* (Askeland and Morrison 1983). HCN is highly toxic to most aerobic microorganisms, because of its ability to block the cytochrome oxidase pathway event at very high dilutions (pmol). Suppression of disease development is attributed to the action of HCN on certain oomycetes. Development of tobacco black rot disease caused by *Thielaviopsis basicola* was suppressed by *P. fluorescens* CHA0 that produced HCN, in addition to other antibiotics and siderophores. The mutants of wild type strain CHA0 deficient in synthesis of HCN, antibiotics, exoenzymes could not protect the tobacco plants against infection by *T. basicola.* HCN was considered to be primarily responsible for the biocontrol of tobacco black rot disease

(Voisard et al. 1989). In *P. fluorescens* strains Q2-87 and CHA0, the *hcnABC* gene encode for HCN synthetase required for HCN production (Haas and Défago 2005). In order to detect the HCN producers in a mixed population of pseudomonads, primers targeting *hcnAB* genes were designed with MultiAlin from the consensus of the *hcn* sequences between *P. fluorescens* strain CHA0 and *P. aeruginosa* strain PAO1 were designed. A single amplicon of about 570-bp in length was obtained for all HCN^+ strains using the PCR procedure developed in this investigation. No amplicon was generated from the two negative HCN pseudomonads. The HCN^+ bacterial strains could be sensitively detected in samples where they were in a low, percentage of total pseudomonad community and/or where the numbers were low regardless of their proportion to the numbers the total community which is an important feature of environmental biodiversity analysis (Svercel et al. 2007).

Cyclic Lipopeptides

Various bacterial genera have been shown to produce peptide antibiotics exhibiting antagonistic activity against a wide range of fungal plant pathogens. Cyclic lipopeptides (CLPs) have been detected in the cultures of both Gram-positive and Gram-negative bacteria. *Pseudomonas* spp. are known to produce CLPs commonly. An antifungal cyclic peptide effective against *Rhizoctonia solani* was isolated from *P. fluorescens* strain 96.578. The production of the CLP tensin was high in liquid media with glucose, mannitol or glutamate as growth substrate. When applied to sugar beet seeds, the strain 96.578 produced tensin during seed germination (Nielsen et al. 2000). Strains of *P. fluorescens* have been reported to produce three types of CLPs viz tensin (Henriksen et al. 2000), amphisin (Sorensen et al. 2001) and viscosinamide. The CLP antibiotics have either 9 or 11 amino acids in the peptide ring. They possess antimicrobial and biosurfactant properties (Nielsen et al. 2002). The low molecular weight biosurfactants include glycolipids and lipopeptides such as rhamnolipids and surfactin. Rhamnolipids secreted by strains of *Pseudomonas aeruginosa* were effective against *Pythium aphanidermatum, Plasmopara lactucae-radicis* and *Phytophthora capsici*. Purified rhamnolipids caused cessation of motility and lysis of entire zoospore populations within <1 min (Stanghellini and Miller 1997). Rhamnolipid B produced by *P. aeruginosa* B5 inhibited the mycelial growth of *Phytophthora capsici* and spore germination of *Colletotrichum orbiculare* in in vitro assays. The diseases caused by these pathogens were suppressed in pepper and cucumber respectively following application of purified rhamnolipid B (Kim et al. 2000). *Pseudomonas fluorescens* strain SS 101 (biovar II) isolated from wheat rhizosphere exhibited zoosporicidal activities rendering the zoospores of *Pythium* spp. *Phytophthora infestans* and *Albugo candida* inactive. Application of cell suspension of strain SS101 to soil or hyacinth bulbs protected the plants against root rot disease caused by *Pythium intermedium*. Two genes involved in surfactant production by *P. fluorescens* SS101 were identified by random Tn5 mutagenesis followed by anchored-PCR and subsequent sequencing of the Tn5 flanking regions. The surfactants produced by strain SS101 were isolated by reverse-phase high-pressure liquid chromatography (RP-HPLC). The principal constituent was identified as a

cyclic lipopeptide (1,139-Da) containing nine amino acids and a ten-carbon hydroxyl fatty acid by mass spectrometry and nuclear magnetic resonance analysis. The results suggest that the biosurfactants produced by the strain SS101 might play a key role in the biocontrol activity against *P. intermedium* (de Souza et al. 2003a, b).

Cyclic lipopeptides (CL) produced by bacterial species have been reported to be responsible for their biocontrol activities against fungal pathogens. A derivative of *Bacillus subtilis* BBG100 that over-produced the CLP mycosubtilin exhibited enhanced activity against *Pythium* spp. infecting tomato seedlings (Leclére et al. 2005). Application of viscosinanmide-producing *P. fluorescens* strain DR54 to sugar beet substantially increased plant emergence and root length in soil infested with *Pythium ultimum* (Thrane et al. 2000). Production of CLPs with antifungal and biosurfactant properties by *Pseudomonas fluorescens* strains in bulk soil and sugar beet rhizosphere was investigated. When sugar beet seeds were coated with CLP-producing strains and subsequently germinated in non-sterile soil, the strain DR54 maintained a high and constant viscosinamide level in young rhizosphere for about 2 days, while strains 96.578 and DSS73 produced higher concentrations of the CLPs tensin or amphisin. All three CLPs were present in detectable levels for several days in rhizosphere. The results suggested that production of CLPs might occur only in specific habitats like rhizosphere of germinating sugar beet seeds rather than in the bulk soil (Nielsen and Sørensen 2003). In another study, production of an antifungal polyketide 2,3-deepoxy-2,3-didehydrorhizoxin (DDR) by *Pseudomonas* sp. effective against wheat seedling blight disease caused by *Fusarium culmorum*, was observed (Johansson and Wright 2003).

Strains of *Pseudomonas fluorescens* have been reported to produce surface-active compounds called as surfactants with inhibitory effects on zoospores of *Phytophthora infestans* and *Pythium* spp. included in Oomycetes (de Souza et al. 2003a, b; De Bruijn et al. 2007). The biosurfactant produced by *P. fluorescens* strain SS101 was identified as massetolide A, a cyclic lipopeptide with 9-amino acid peptide ring linked to 3-hydroxydecanoic acid (de Souza et al. 2003a, b). Application of the strain SS101 suppressed effectively the Pythium root rot infection of flower bulb crops (de Boer et al. 2006). The role of massetolide A in the biocontrol activity of the strain SS101 was investigated. The gene *massA* governing the biosynthesis of the surfactant massetolide has been identified in *P. fluorescens* SS101. The capacity of the wild-type strain and its mutant or the cyclic lipopeptide surfactant massetolide A to inhibit hyphal growth of different *Pythium* spp. was assessed in vitro. Strain SS101 only marginally suppressed in vitro growth of *Pythium* spp. and growth was not inhibited in the presence of the mutant 10.24. The results indicated that *P. fluorescens* SS101 was very effective in controlling diverse *Pythium* populations infecting different crops grown in various soils. Further, production of the cyclic lipopeptide massetolide A did not have significant role in suppression of root rot disease (Mazzola et al. 2007). *Pseudomonas fluorescens* SS101 effectively prevented infection of tomato leaves by the late blight pathogen *Phytophthora infestans* and also restricted the expansion of existing lesions and sporangial production. As the sporangia form an important primary

and secondary inoculum source for *P. infestans*, destructive effect of strain SS101 on both lesion area and sporangia formation might lead to a reduction in rate of disease development. This investigation showed that massetolide A-deficient mutant 10.24 was significantly less effective in its biocontrol activity, compared with wild-type strain. Application of massetolide A on tomato leaves and roots effectively protected treated plants against *P. infestans*. These findings showed that the CLP massetolide A was an important component of the biocontrol efficacy of strain SS101. The purified preparation of massetolide A produced significant control of the tomato late blight disease both locally and systemically via induced resistance indicating the multifunctional capacity of the cyclic lipopeptide produced by *P. fluorescens* SS101 (Tran et al. 2007).

The biosurfactants are capable of affecting cell surface of plant pathogenic fungi (Raaijmakers et al. 2006) and also have the ability to act on lipids creating pores on the membrane layer (Kim et al. 2004a, b). The role of biosurfactants in the *Pseudomonas*-mediated reduction in the viability of microsclerotia of *Verticillium longisporum* infecting cauliflower, was investigated. The biosurfactant deficient mutants of *Pseudomonas* (MR 12a) and *P. aeruginosa* PNA1 were less effective in the suppression of the viability of *Verticillium* microsclerotia, when compared with the wild-type strain. The biosurfactants were effective at a bacterial cell density of 2×10^9 CFU/ml. The results showed that biosurfactant production by BCA did not fully account for *Verticillium* microsclerotia suppression. Other mechanisms may also be involved in the suppression of microsclerotial germination. The biosurfactant mutant of *P. aeruginosa* PNA1 had residual effect on the formation of secondary microsclerotia. Likewise, the biosurfactant of *Pseudomonas* CMR12a had a residual effect on *Verticillium* germination and the formation of secondary microsclerotia. The residual adverse effects of the mutants were attributed to their ability to produce the antibiotic phenazine-1-carobxylic acid (PCA) (Debode et al. 2007). *Pseudomonas* sp. DF41 suppressed effectively the stem rot disease of canola caused by *Sclerotinia sclerotiorum*. DF41 strain produced a number of compounds including hydrogen cyanide (HCN), protease, alginate and lipopeptide (LP) molecules. All these compounds that might contribute to the biocontrol potential of DF41 were under the control of Gac. DF1 also produced autoinducers, suggesting that the bacterium employed quorum sensing as part of its lifestyle. Two mutants of DF41 strain with drastically reduced antifungal activity were generated by transposon mutagenesis technique. The *gacS* (DF41-469) mutant had an insertion in *gacS* forming part of the GacS/GacA regulatory system, while the *lp* (DF469-1278) mutant had an insertion in lipopeptide synthesis. The *gacS* and *lp* mutants could not protect canola plants against *S. sclerotiorum* in the greenhouse tests. Both mutants could not persist in canola phyllosphere. The loss of biocontrol activity of the mutants was considered to be due to the reduced synthesis of antifungal compounds and not due to a declining population size. It was concluded that the strain DF41 relied on the production of antifungal compounds (LPs) for suppressing the development of Sclerotinia stem rot disease of canola (Berry et al. 2010).

The possibility of using biosurfactants produced by bacterial biocontrol agents for suppressing the development of zoospore-producing oomycetes and the diseases caused by them especially in closed hydroponic cultivation systems was explored. The potential of a biosurfactant produced by *Pseudomonas koreensis* was assessed by applying a crude extract of the BCA. Assessment of the effect of the crude extract on *Pythium ultimum* in hydroponic tomato cultivation showed that incidence of the disease was significantly reduced. Application of the biosurfactant did not influence the indigenous microflora, when evaluated as sole carbon source utilization (Hultberg et al. 2010a). The efficacy of the biosurfactant from *P. koreensis* the strain 2.74 against the potato late blight pathogen *Phytophthora infestans* was assessed using a detached leaf assay. Development of disease on the treated leaves challenged with the pathogen zoospores was reduced significantly. The biosurfactant inhibited the motility of the pathogen zoospores, but caused only a minor reduction in mycelial growth rate. No adverse effect on the rate of sporangia production in pure culture was observed due to treatment with the bacterial biosurfactant (Hultberg et al. 2010b). Use of antagonistic microorganisms for the control oomycetes infecting crops in hydroponic systems is a sustainable approach, since the biosurfactans and biosurfactant-producing microorganisms have been found to be potentially useful components of a sustainable biocontrol strategy. Three modes of supplying the biosurfactant-producing strain to a recirculating hydroponic cultivation system infected with the zoospore producing pathogen, *Pythium ultimum* were evaluated. *Pseudomonas koreensis* strain 2.742 was added as washed cells, in its spent KB broth or in a minimal medium adapted from the nutrient solution and compared with control treatments. When a high concentration of washed cells of the strain 2.74 was added to the plant cultivation system, the infection was reduced up to 50 %. Addition of purified biosurfactant also protected the tomato plants as effectively as the washed cells of the BCA strain. The spent broth induced phytotoxic symptoms. The results indicated the effectiveness of the bacterial strain as well as the purified biosurfactant for the management of disease caused by zoospore-producing oomycetes (Hultberg et al. 2011).

Pseudomonas aeruginosa PNA1 isolated from chickpea rhizosphere has been shown to be effective against *Pythium* spp. and other pathogenic fungi. *P. aeruginosa* PNA1 produces rhamnolipid biosurfactants, in addition to phenazines. The biosurfactants and the antibiotics produced by the strain PNA1 might act synergistically. Phenazines and surfactants might interact with the mycelium and zoospores respectively, the two important asexual propagules of *Pythium* spp. (de Souza et al. 2003a, b). The role of biosurfactants in the biocontrol activity of *P. aeruginosa* PNA1 was investigated in vivo via mutant analysis and by using purified surfactants. A rhamnolipid-deficient and a phenazine-deficient mutant of PNA1 were used either separately or jointly in the greenhouse. The experiments on cocoyam (*Xanthosoma sagittifolium*) and bean (*Phaseolus vulgaris*) demonstrated the importance of biosurfactants for the biocontrol of *P. myriotylum* and *P. splendum,* infecting cocoyam and bean respectively. Lysis of zoospores was caused by the surfactants, due to intercalation with the zoospore plasma membranes which are not protected by a cell wall as in the case of other fungal cells. When the mutants were concurrently introduced in the

soil, the biocontrol activity was restored to the wild-type levels. When bean seeds were treated with a mixture of purified phenazine-1-carboxamide (PCN) and rhamnolipids, pre-emergence damping-off was more effectively reduced, than when the seeds were treated with either of these compounds alone. The results indicated the synergistic activity of PCN and rhamnolipids, resulting in more effective disease suppression (Perneel et al. 2008).

Antibiotics have been demonstrated to play a significant role in crop disease management by employing the bacterial biocontrol agents. In the recent years, new insights and concepts in biological control of plant pathogens by bacteria producing antibiotics in different substrates have been developed. Assays performed in vitro provided data on broad-spectrum activity of most bacterial BCAs Different lines of evidences supported the role and functions of antibiotics in in situ interactions between the bacterial BCAs and microbial plant pathogens. Culture filtrates or purified antibiotics provided similar levels of suppression of pathogen growth or disease development. Inactivation of antibiotic production by mutagenesis led to a reduction in the biocontrol capacity of the bacterial strain. It was possible to enhance the level of antibiotic production in the wild-type strain via introduction or modification of antibiotic biosynthetic or regulatory genes. Another line of evidence was provided by introduction of antibiotic biosynthetic genes in heterologous, non-producing strains and subsequent evaluation of their ability to control plant diseases. Reporter gene systems and bio-analytical techniques have clearly shown the presence of antibiotics secreted by bacterial BCAs in the spermosphere and rhizosphere of a range of host plants. Despite the availability of several evidences of the presence of antibiotics in the substrates of interaction of BCA-pathogen, the question as to whether the amount of antibiotic produced, is sufficient to inhibit the growth or metabolic activity of the pathogen in situ is yet to be addressed.

Furanones

Fungi, bacteria and actinomycetes have been reported to produce 3-(1-hydroxyalkyl)-2 (5H)-furanones with antimicrobial properties (Nagnuma et al. 1992; Ordentlich et al. 1992; Braun et al. 1995). *P. aureofaciens* strain 63–28 produced three 3-(alkyl)-2 (5H)-furanone derivatives which inhibited the radial growth of *Pythium ultimum* and induced abnormal appearance of the hyphae (Paulitz et al. 2000). *Pseudomonas jessenii* produced two secondary metabolites viz (i) 3-[(1R)-hydroxy-octyl]-5-methylene-2(SH)-furanone (4,5 didehydraacaterin) and (ii) 3-[(1R)-hydroxyhexyl]-5-methylene 2(5H)-furanone that induced hyperbranching and swelling of hyphae of *Aphanomyces cochlioides* and *Pythium aphanidermatum*. Treatment of the hyphae of these fungal pathogens with the two furanones produced by *P. jessenii* caused characteristic accumulation of plaques at the hyphal tips, similar to the effects induced by an actin assembling inhibitor. Staining with rhodamine-phalloidin which binds to plasma membrane-associated filamentous-actin (F-actin) revealed that tip-specific actin filaments were remodelled into a plaque-like form at an early stage of encounter (up to 24 h) with the furanones. At later stages of encounter (48 h), the plaques were eliminated reflecting the disorganization of actin arrays in the morphologically

abnormal hyphae of both pathogens, as observed under confocal laser scanning microscope (CLSM) and transmission electron microscopic (TEM). The results suggested that the mechanism of action of furanones on fungal pathogens may be through interference in morphogenesis and growth establishment and maintenance of cell polarity and subcellular organization of the pathogens and these alterations may be mediated by F-actin (Deora et al. 2010).

Volatile Organic Compounds

The bacterial biocontrol agents *Pseudomonas fluorescens* Q8r1-96, *P. fluorescens* B-4117 and *Serratia plymuthica* IC1270 have been shown to significantly suppress the tumor development induced by *Agrobacterium vitis* in tomato seedlings which were treated with the BCAs prior to pathogen inoculation (Khmel et al. 1998; Raaijmakers and Weller 2001; Ovadis et al. 2004). The mutants deficient in 2,4-diacetylphloroglucinol (2,4-DAPG) and pyrrolnitrin (Prn) production were also as effective as the wild-type strains, indicating that the antibiotics produced by these BCAs are not involved in tumor development suppression. In order to identify the compounds of BCA origin involved in the biocontrol activity against *A. vitis,* the volatile organic compounds (VOCs) produced by the three strains of BCAs were evaluated for their efficacy in inhibiting the growth of *A. vitis* and suppressing tumor development in tomato. The VOCs produced by strains of *P. fluorescens* and *S. plymuthica* inhibited the growth of *A. tumefaciens* and *A. vitis* strains in vitro. A derivative of *P. fluorescens* Q8r1-96 tagged with a *gfp* reporter and *P. fluorescens* B-4117 and *S. plymuthica* IC1270 marked with rifampicin resistance stably persisted in tomato tissues for at least 1 month. Solid-phase micro-extraction-gas chromatography–mass spectrometry analysis revealed that dimethyl disulfide (DMDS) was the major headspace volatile produced by *S. plymuthica* and it was able to suppress the growth of *Agrobacterium* effectively in vitro. In addition, DMDS was emitted by tomato plants treated with *S. plymuthica*. On the other hand, *P. fluorescens* strains produced 1-undecene as the main volatile compound and the volatiles including DMDS were produced in relatively low quantities (Table 5.3) (Dandurishvili et al. 2010) (Appendix 5.3).

The mechanisms underlying the VOCs action could be related to the ability of some volatiles to interfere with the levels and ratios of phytohormones (Zhang et al. 2007) which are known to be important factors in crown gall formation. Volatiles produced by some rhizobacterial strains trigger growth promotion and induce systemic resistance in plants through cytokinin and ethylene signaling pathways (Ryu et al. 2004; Farag et al. 2006). Inhibition of ethylene-signaling pathway might be effected by PGPR-by producing the enzyme 1-aminocylopropane-1-carboxylate (ACC) deaminase which can degrade ACC-the immediate precursor of ethylene in plants. This may result in lower plant ethylene levels and consequently suppression of crown gall formation. The possibility of DMDS and/or other VOCs being involved in inducing systemic resistance to crown gall disease in tomato has to be investigated (Narayanasamy 2008).

Table 5.3 Inhibition of growth of *Agrobacterium* spp. by strains of *Pseudomonas fluorescens* and *Serratia plymuthica* (Dandurishvili et al. 2010)

	Antagonist strain				
Pathogen strain[b]	B-4117	Q8r 1-96	Q8r 1–96 mutant (DAPG$^-$)	IC 1270	IC 1270 mutant (Prn – 1)
At C58	16–20[a]	6–10	nz[b]	6–10	nz
At Sh-1	11–15	6–10	nz	11–15	1–5
Av S4	11–15	6–10	1–5	11–15	nz
AvTm4	11–15	6–10	nz	11–15	nz

[a]Growth inhibition zone (mm)
[b]At *Agrobacterium tumefaciens*, AV *A. vitis*, nz no inhibition zone

Bacteriocin-Mediated Antagonism

Bacteriocins secreted by bacterial species are proteinaceous compounds with deleterious effects on closely related bacterial competitors in the same substrate/environment. These toxic metabolites constitute a structurally and functionally diverse group within the antimicrobial compounds. *Pseudomonas* spp. colonizing rhizosphere have been reported to produce bacteriocins. The bacteriocin LipA produced by *Pseudomonas* sp. strain BW11M1 is a novel type of antibacterial protein and it exhibits homology to mannose-binding lectins mostly found in monocots (Parret et al. 2003). The biocontrol agent *P. fluorescens* strain Pf-5 has two *llpA*-like genes designated *llpA1-p$_f$-5* land *llpA2p$_f$-5*. Recombinant *Escherichia coli* cells expressing *llpA1pf-5* or *llpA2pf-5* acquired bacteriocin activity and secreted a 31-kDa protein cross-reacting with $LlpS_{BWIIMI}$ antibodies. Analysis of antimicrobial spectrum showed that $LlpA1_{pf\text{-}5}$ and $LlpA2p_{f\text{-}5}$ were able to inhibit *P. fluorescens* strains. Bacteriocin production by BCAs may contribute to their rhizosphere competence. Thus the recombinant strains equipped with bacteriocin genes may be able to perform better as plant growth-promoting inocula by out-competing rhizosphere inhabiting pseudomonads (Parret et al. 2005).

Enzyme-Mediated Antagonism

Strains of *Pseudomonas* spp. may be able to suppress crop diseases by producing antibiotics, lytic enzymes and siderophores, by depriving pathogens of nutrients and habitation sites or by inducing systemic defence reactions in the host plants (Leong 1986; Cook 1993). Chitinase, a hydrolytic enzyme capable of degrading fungal cell-wall components is one of the mechanisms implicated in biocontrol (Ordentlich et al. 1998). Genetically engineered BCAs with multicopy chitinase genes have been demonstrated to have greater pathogen suppressive capacity (Sundheim et al. 1988; Simi 1994). Inhibition of *R. solani* was significantly enhanced, when *P. fluorescens* transformed with chitinase gene *chiA*. The extent of inhibition of mycelial growth was correlated with level of suppression of damping-off disease of cotton (Simi 1994). Likewise, *Pseudomonas* strain NRR1 B-15135 expressing heterogenous

chitinase genes from *Serratia marcescens* possessed greater level of biocontrol capacity against radish Fusarium wilt. However, no improvement in the disease suppressive effect could be observed against wheat take-all disease (Sundheim et al. 1988). Antagonism of *Pseudomonas* spp. against microbial plant pathogens has been observed only in a few cases. Involvement of a glucanase β-1,3-glucanase produced by *P. cepacia* in suppressing the development of *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* was able to degrade and lyse fungal mycelia. The bacterial enzymes chitinases and β-1,3-glucanase are encoded by a single gene (Siddique 2005).

Necrosis-inducing plant pathogenic pseudomonads produce two groups of cylic lipodepsipeptides (LDPs) that are toxic to plants and microorganisms. One group contains nonapeptide lactones like syringomycins (SRs), syringtoxins (STs), syringostatins and pseudomycins. The other group with lipopeptides includes tolaasins, syringopeptins (SPs) and fuscopeptins. *Pseudomonas syringae* pv. *syringae* (*Pss*) secreted two main LDPs, syringomycin E (SRE) and syringopeptin 25A (SP_{25A}) together with at least four types cell wall-degrading enzymes (CWDEs). In antifungal bioassays, the purified toxins SRE and SP_{25A} interacted synergistically with chitinolytic and glucanolytic enzymes purified from the same bacterial strain or the fungal BCA *Trichoderma atroviride* strain P1. The synergism between LDPs and CWDEs occurred against fungal pathogens *Verticillium dahliae, Botrytis cinerea*, *Penicillium expansum* and *Phytophthora infestans* as well as *P. syringae* itself, with a level dependent on the enzyme used to permealize the microbial cell wall. The in vivo biocontrol assays using *P. syringae* alone or in combination with *T. viride*, including a *Trichoderma* endochitinase knock-out mutant indicated that the synergistic interaction between LDPs and CWDEs was involved in the antagonistic mechanisms of biocontrol activity may be more effective in suppressing development of crop disease (Fogliano et al. 2002). *Pseudomonas fluorescens* strain P5 showed strong inhibitory effect on *Gaeumannomyces graminis* var. tritici causing wheat take-all disease and *Rhizoctonia solani* causing damping-off disease of cotton. The strain P5 was transformed with the 6.5-kb chitinase gene fragment from the bacterial BCA *Serratia marcescens* strain M90-3. The biocontrol potential of the wild type strain P5 and the transformant P5-1 was compared by both in vitro and in vivo tests. Inhibition of mycelial growth of *G. graminis* var. *tritici. R. solani,* infecting cotton (damping-off disease) and *R. solani* infecting rice (sheath blight disease) by the transformant P5-1 was significantly more than that of the wild-type strain P5. Both the wild-type and transformant strains suppressed the development of wheat take-all, cotton damping-off and rice sheath blight diseases. However, the transformant P5-1 protected the cotton and rice plants more effectively against *R. solani* than the wild-type strain (Table 5.4) (Xiao-Jing et al. 2005).

Groundnut (peanut) seed endophyte *Pseudomonas aeruginosa* strains GSE 18 and GSE19 reduced the incidence of stem rot disease caused by *Sclerotium rolfsii* and the seedling mortality by 54 and 58 % respectively. In dual cultures, these strains reduced the mycelial growth of *S. rolfsii*. Cell-free culture filtrates of the strains GSE18 and GSE19 inhibited the activity of the cell wall-degrading enzymes (CWDEs) polygalacturonase and cellulase produced by *S. rolfsii* to the maximum

Table 5.4 Biocontrol efficacy of wild-type and transformed strains of *Pseudomonas fluorescens* in suppressing development of diseases in wheat, rice and cotton (Xiao-Jing et al. 2005)

		BCA strain	
Disease/effect of BCA	Control	P5	P5-1
A. Wheat take-all			
Disease index	42.3 ± 5.9a	26.4 ± 3.3b	21.2 ± 3.6b
Control effect (%)	–	37.6	49.9
B. Rice sheath blight			
Lesion length (cm)	6.21 ± 0.12a	4.02 ± 0.18b	3.14 ± 0.15c
Control effect (%)	–	35.3	49.4
C. Cotton damping-off			
Dead plants(%)	80.1 ± 4.6a	64.2 ± 2.7b	55.3 ± 3.2c
Control effect (%)	–	19.8	31.0

Values are mean ± standard deviation for 12 replicates; for each disease, values followed by the same letter are not statistically significant ($P < 0.01$)

extent of 55 and 50 % respectively at 6 days after inoculation. As the strains GSE18 and GSE19 were tolerant to thiram, a commonly applied seed dressing fungicide, the potential of *P. aeruginosa* strains for the biocontrol of stem rot disease has to be exploited (Kishore et al. 2005a, b). In another study, the strong antagonistic activity of *P. aeruginosa* strain GRC against *Sclerotinia sclerotiorum* causing stem rot disease of groundnut was observed. Scanning electron microscopic (SEM) observations revealed morphologic abnormalities such as perforation, lysis and fragmentation of hyphae of *S. sclerotiorum* induced by *P. aeruginosa*. The strain GRC secreted extracellular chitinase. The role of the chitinase in suppressing the mycelial growth was demonstrated through *Tn5* mutagenesis. Seed bacterization with strain GRC improved seed germination and reduced stem rot disease incidence in pathogen-infested soil by 97 %. In addition, plant growth, number of nodules, pods and grain yield/plant were significantly increased in comparison to control (Gupta et al. 2006).

The biocontrol efficacy of a bacterial species may be enhanced by the presence of another bacterial species producing fungal cell wall-degrading enzymes. *Pseudomonas fluorescens* strain LRB3W1 suppressed the development of many plant diseases including cabbage Fusarium yellows disease caused by *Fusarium oxysporum* f.sp. *conglutinans* (FOC) by producing antibiotics (Someya et al. 2007b) A mycolytic bacterial species *Serratia marcescens* strain B2 inhibited several fungal pathogens, causing diseases such as cucumber damping-off, gray mold disease and rice sheath blight (Someya et al. 2005a, b). The strain B2 produced chitinases, a class of cell wall-degrading enzymes (Someya et al. 2001). *P. fluorescens* strain LR3W1 inhibited the mycelial growth of FOC, while *S. marcescens* strain B2 did not affect the pathogen growth. The disease suppressive effect of strain B2 was less than that of strain LRB3W1. But the combined effect of both bacterial strain was much more greater than strain LRB3W1 alone. Colonization of each bacterial strain was not influenced by one another. Treatment of bud cells of FOC with 2,4-DAPG from strain LRB3W1 and fungal cell wall-degrading enzyme from strain B2 resulted in an additive adverse effect. The results indicated the synergistic action of strain B2

leading to enhancement of effectiveness of suppression of disease caused *F. oxysporum* f.sp. *conglutinans* in cabbage (Someya et al. 2007a).

Pseudomonas chlororaphis strain PA-23 was effective in suppressing the development of the canola stem rot disease caused by *Sclerotinia sclerotiorum.* The fungal pathogen produces ascosporic inoculum from germinating sclerotia and the ascospores infect the flowers of canola. Chitinase and β-1,3-glucanase are the key enzymes involved in the lysis of pathogen cell walls. The ability of the strain PA-23 to induce the activities of these two enzymes was assessed by spraying plants twice at 30 and 50 % bloom followed by challenge inoculation with *S. sclerotiorum.* The BCA-treated canola plants had significantly higher chitinase and β-1,.3-glucanase activities. On the other hand, the activities of these enzymes were less in healthy control, ascospore-inoculated control and PA-23-treated plants. β-1,.3-glucanase attained the peak at 4 days after inoculation (DAI) for chitinase and at 6 days of β-1,.3-glucanase. The enzymatic activities declined slowly with increase in time (Fernando et al. 2007). The impact of treatment with the root nodulating *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 on plant growth and suppression of the development of the pigeonpea wilt disease caused by *Fusarium udum* was studied. Both bacterial strains showed chitinase and β-1, 3-glucanase activities and also produced siderophore, indoleacetic acid (IAA), and solubilized insoluble phosphate. In addition, the bacterial strains could degrade and digest cell wall components resulting in hyphal perforations, empty cell (halo) formation, shrinking and lysis of fungal mycelia along with significant degeneration of conidia. The mycelial growth of the pathogen *F. udum* was drastically arrested. Furthermore, *P. fluorescens* LPK2 produced volatile cyanogens (HCN) toxic to the pathogen. In addition to disease suppression, bacterial inoculants, promoted seed germination, and enhanced number of nodules formed, shoot and root length, shoot and root weight, and number of pods, when they were applied with half-dose of chemical fertilizers. The results showed that the strains LPK2 and KCC5 exhibited synergism, aggressive colonization of the roots, enhancement of plant growth and greater efficiency in suppressing the development of pigeonpea wilt disease (Kumar et al. 2010).

5.1.1.2 Competition for Nutrients and Space

Siderophore Production

Bacterial BCAs present in different substrates compete for the available nutrients with the microbial plant pathogens and other microorganisms. Availability of essential micronutrients such as iron is a crucial factor for all microorganisms. Iron becomes a limiting factor in the rhizosphere depending on the soil pH. In highly aerated (oxidized) soil, iron present in ferric form is insoluble in water and in low concentration ($<10^{-18}$ M). Hence, it may not be available to the microorganisms. The iron-binding ligands known as siderophores with high affinity for iron are produced by all microorganisms to sequester iron from the micro-environment.

Ability to produce siderophores confers to bacteria competitive advantages to colonize plant tissues and to exclude other microorganisms from the same ecological niche. The siderophores may be of two types: catechol type or hydroxamate type (Neilands 1981). The fluorescence of pseudomonads is attributed to the presence of an extracellular diffusible pigment called pyoverdin (Pvd) or pseudobactin. This pigment has high affinity for Fe^{3+} ions and it is a siderophore (iron-carrier) of the producer strain. In iron-depleted media, Pvd-producing *Pseudomonas* spp. are able to inhibit the growth of bacteria and fungi with less potent siderophores. Under certain conditions, Pvd functions as a diffusible, bacteriostatic or fungistatic antibiotic. It may be possible for a sessile producer of potent siderophore like Pvd might compete at a distance with other microorganisms that have less efficient iron-uptake systems (Kloepper et al. 1980).

The importance of production of siderophore as a mechanism of biological control of *Pseudomonas fluorescens* strains A1, BK1, TL3B1 and B10 against the fire blight pathogen *Erwinia amylovora* was demonstrated by Kloepper et al. (1980). Synthesis of siderophore in vitro in *Pseudomonas* spp. was correlated with the capacity to inhibit germination of chlamydospores of *Fusarium oxysporum* (Sneh et al. 1984; Elad and Baker 1985). Under greenhouse conditions, *P. putida* strain B10 suppressed Fusarium wilt and take-all diseases. However, the suppressive effect of the bacteria was lost, when the soil was amended with iron which repressed siderophore production in this strain (Kloepper et al. 1980). In another study, *Pseudomonas* strain B324 with characteristics intermediate between *P. fluorescens* and *P. putida* was inhibitory to all seven isolates of *Pythium ultimum* var. *sporangiferum* infecting wheat, in addition to induction of strong growth-promoting effect on wheat plants under iron-limiting conditions (Becker and Cook 1988). As it can be expected, the mutants deficient in the production of siderophores like pyoverdin showed reduced biocontrol potential in suppressing the development of plant diseases as in the case tobacco black root rot disease by strain *P. fluorescens* CHA0 (Keel et al. 1989).

Bacterial isolates (216) obtained from paddy field soil samples were tested for their siderophore production and effectiveness in inhibiting mycelial growth of *Alternaria* sp. (leaf spot), *Fusarium oxysporum* (root rot), *Magnaporthe grisea* (blast) and *Sclerotium* sp. (stem rot) infecting rice. In dual culture technique, siderophore-producing rhizobacteria showed strong antagonistic activity against all the four rice pathogens to varying degrees ranging from 10.4 to 37.5 %. *Pseudomonas aureofaciens* strain AR1 was the most effective producer of siderophore and it secreted hydroxymate type siderophore. Siderophore in the strain AR1 reached the peak after 15 days at an optimal temperature of 30 °C, yielding 99.96 ± 0.46 μg/ml of siderophore. This study showed selection of bacterial isolate capable of producing siderophores, in addition to their ability to function via other mechanisms of biocontrol activity to achieve greater effectiveness in disease management (Chaiharn et al. 2009). *Pseudomonas aeruginosa* (PN1 ~ PN10) strains (10) isolated from rhizosphere of chir-pine were evaluated for their antagonistic activities against the root rot pathogen *Macrophomina phaseolina* and plant growth properties in vitro and in vivo. *P. aeruginosa* PN1 produced siderophore, indole-acetic acid (IAA), cyanogen and solubilized

phosphorus, in addition to chitinase and β-1, 3-glucanase. In dual culture assay, the mycelial growth of *M. phaseolina* was inhibited by 69 %. The culture filtrate was less effective in inhibiting the mycelial growth of the pathogen. *P. aeruginosa* PN1 increased the plant growth and biomass in pot experiment containing the pathogen-infested soil. PN1 showed the strong chemotaxis toward root exudates resulting in effective root colonization. The BCA strain exhibited strong antagonistic property against *M. phaseolina,* suppressed the development of disease in chir-pine and improved growth of chir-pine seedlings (Singh et al. 2010).

The genetic diversity of siderophore-producing bacteria of tobacco rhizosphere was assessed by applying amplified ribosomal DNA restriction analysis (ARDRA), 16S rRNA sequence homology and phylogenetic analysis. Bacteria belonging to 14 different genera generated 28 ARDRA patterns. Gram negative isolates were more frequently detected with more than 95 % total frequency. For Gram-positive bacteria, *Bacillus* and *Rhodococcus* were the only two genera with a 1.7 % total frequency. *Pseudomonas* and *Enterobacter* isolates were dominant in the tobacco rhizosphere environment with 44.5 and 24.7 % total frequency respectively. It was observed that 75 % of the isolates that had the high percentages of siderophore units (between 40 and 60 %) belonged to *Pseudomonas* spp. The G-229-21 strain of *Pseudomonas* could produce high-affinity carboxylate type siderophores under low iron-conditions and its siderophores strongly suppressed the development of *Phytophthora parasitica* var. *nicotianae* under low iron conditions (Tian et al. 2009). The diversity of endophytic siderophore-producing bacteria (SPB) associated with rice plants was studied. The presence of the SPB species was detected in grains, leaves and roots. The population of the heterotrophic siderophore producing bacteria was higher in mature rice plants. The amplified restriction DNA ribosomal analysis (ARDRA) was applied to generate patterns specific to different bacterial genera belonging to *Pseudomonas, Sphingomonas, Burkholderia* and *Enterobacter.* Among the bacterial isolates tested, only one isolate of *Pseudomonas* was able to inhibit *Azospirillum brasilense* and *Herbaspirillum seropedicae*. *Pantoea ananatis* was the most common species associated with roots at tillering and leaves to subsequent stages (Loaces et al. 2011).

Pseudomonas fluorescens strain A506, a commercially available product (marketed as Blight Ban A506) is recommended for the control of fire blight disease affecting apple and pear trees. The strain A506 has to outcompete *Erwinia amylovora,* causative agent of fire blight disease, for sites and nutrients for epiphytic growth and subsequent infection of the host plant. The bioavailability of iron on floral surfaces is a crucial factor that influences the production of antibiotics effective against *E. amylovora.* An iron biosensor was used to examine the relative bioavailability of iron to A506 (*pvd-inaZ*) on surfaces of pear and apple flowers through the suppression or expression of an iron-regulated promoter at the transcriptional level. The strain A506 produced a pyoverdin siderophore on iron-depleted media, but not in media amended with 0.1 mM ferric chloride. Concentration of iron that suppressed pyoverdin production also stimulated antibiosis. The results indicated that chelated forms of iron could be oversprayed on flowers colonized by the strain A506 or mixed with the inoculum to increase iron bioavailability to the bacterial

BCA on the surface of pear or apple flowers (Temple et al. 2004). *P. fluorescens* strain CV6 effective against *Phytophthora drechsleri* causing cucumber root rot disease produced substantial amount of siderophore, in addition to antifungal compounds and indole-acetic acid (IAA). This strain also produced enzymes involved in disease resistance, indicating the multi-mechanisms of biocontrol activity of the BCA strain (Maleki et al. 2010).

Interactions between rhizosphere microorganism may have positive or negative effect on the extent of disease suppression achieved. Positive interaction between strains of BCAs may be exploited to enhance the effectiveness of biocontrol of plant diseases. *Pseudomonas putida* WCS 358 effectively suppressed radish Fusarium wilt disease caused by *F. oxysporum* f.sp. *raphani* by competing for iron through the production of the siderophore pseudobactin. Addition of iron to the nutrient solution for plants reduced the disease suppressive effect of the strain WCS358. The pseudobactin negative mutants of WCS358 could also suppress the wilt disease as efficiently as the parent wild-type strain, suggesting that the mutants might act on the pathogen through an alternative mechanism. *P. putida* strain RE8 did not rely on the production of pseudobactin for its disease-suppressive activity. The strain RE8 was found to induce systemic resistance in treated plants. When the mixture of the strains WCS 358 and RE8 was applied to the soil, the effectiveness of disease suppression was enhanced to higher level, compared to treatments with single strain. This additive effect on disease suppression could be the result of pseudobactin-mediated competition for iron combined with induced systemic resistance. The results indicated that the strains that could complement with each other by acting through different mechanisms, have to be selected to achieve more effective disease control (de Boer et al. 2003). *Pseudomonas corrugata* causes vein necrosis disease of tomato grown in hydroponic system. A rifampicin resistant mutant named as *Pseudomonas* sp. LSW25R was antagonistic to *P. corrugata* and inhibited the mycelial growth of several fungal pathogens such as *Botrytis cinerea, Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Blossom-end rot (BER) is a physiological disorder affecting tomato fruits. In hydroponically-grown tomato BER was dramatically reduced by 51 %, following treatment with LSW25R. Analysis of leaves from treated plants revealed that calcium uptake was at higher rate, compared with control in the hydroponic system. LSW25R strain could successfully colonize the rhizosphere during cultivation due to its broad-spectrum of antifungal activity and endophytic colonization. Locations of colonization by the strain LSW25R were investigated, using scanning electron microscope (SEM). Tomato roots of 3-day old plantlets were examined. Clusters of the bacterial strain colonized the surface of the epidermis in high-density around the natural aperture of the root (Fig. 5.3a) and on the grooved lines of the epidermal cells in the root (Fig. 5.5b). Further, LSW25R colonized under the epidermal cells around natural apertures (Fig. 5.3c, d). In addition, the BER incidence was significantly reduced, presumably through enhancement of calcium uptake (Lee et al. 2010).

Pseudomonas syringae pv. *syringae* (*Pss*) 22d/93, an epiphyte isolated from soybean leaves showed high level of antagonistic activity against the closely related pathogen *P. syringae* pv. *glycinea* (*Psg*) causing soybean bacterial blight disease

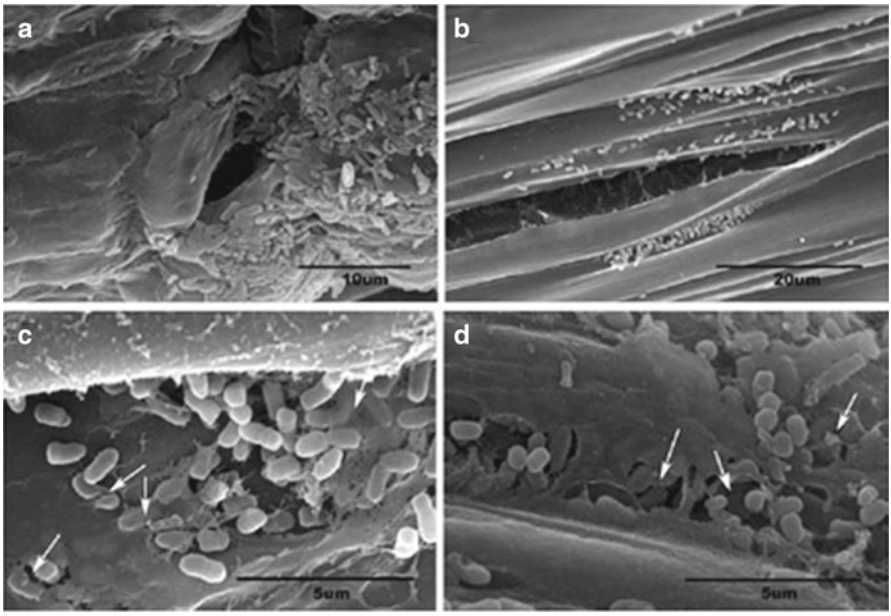

Fig. 5.3 Visualization of colonization by *Pseudomonas* sp. LSW25R of tomato radicle using scanning electron microscope (**a**) and (**b**): clusters of LSW25R localized on the surface of root around root apertures; (**c**) and (**d**): cells of LSW25R present under the epidermis of root (indicated by *arrows*) (Courtesy of Lee et al. 2010 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

(Völksch et al. 1996). The strain Pss22d/93 was able to effectively suppress the bacterial blight disease under field conditions also (Völksch and May 2001). In a later study, *Pss*22d/93 was found to produce significantly larger amounts of siderophores than the pathogen. Both the BCAs produced the same siderophore pyoverdin and achromobactin. However, the regulation of siderophore biosynthesis in pss22d/93 was very different from that of the pathogen. The epiphytic fitness of *Pss*22d/93 mutants defective in siderophore biosynthesis was determined, following spray inoculation of soybean leaves. At 10 days after inoculation, the population of the mutants was reduced by two orders of magnitude lower than that of the wild-type strain. But there was no significant difference in the biocontrol efficacy of the wild-type strain and the mutants of *Pss*22d/93, suggesting that the siderophores did not play any essential role in the biocontrol potential of the strain Pss22d/93. On the other hand, production of pyoverdin and achromobactin contributed significantly to the epiphytic fitness of *P. syringae* pv. *syringae* 22d/93, thus improving the effectiveness of its biocontrol activity against soybean bacterial blight pathogen (Wensing et al. 2010).

Erwinia amylovora, causing the fire blight disease of apple and pear, requires nicotinic acid (NiAc), nicotinamide ($NiNH_2$) and/or 6-hydroxynicotinic acid (6-HNiAc) as essential growth factors, when cultured on minimal medium in vitro (Paternoster

et al. 2009). It may be expected that an organism capable of eliminating or reducing the availability of $NiNH_2$ might effectively inhibit the growth of *E. amylovora* and suppress the development of fire blight disease. A total of 735 bacteria and 1,237 yeast isolates obtained from apple blossoms were pre-screened for nicotinic acid-degradation. The strain JAN degraded NiAc for its growth to the maximum extent and also showed the highest levels of biocontrol efficacy against *E. amylovora.* This strain was identified as *Pseudomonas rhizosphaerae* strain JAN, based on high 16S rRNA gene sequence homology. The JAN mutants M3G7 and M40E5 with impaired capacity to degrade NiAc and $NiNH_2$ showed strong reduction in their biocontrol activity against *E. amylovora.* M3G7 and M40ES were disrupted in *ntrC* and *ntrB* genes respectively. The biocontrol potential of the strain JAN and *P. fluorescens* A506, a commercial product used as a reference strain in experiments was assessed both in vitro and in the greenhouse. The efficacy of JAN on pear slices and apple hypanthia was comparable to that of strain A506. Under greenhouse conditions, JAN showed consistent high level of control across all three experiments, whereas the strain A506 was less effective in two of the three experiments. The results indicated that the bacterial BCA JAN could suppress the development of fire blight disease by effectively degrading the essential growth factor of the pathogen *E. amylovora* (Paternoster et al. 2010).

Competition between the biocontrol agents and the pathogens for available nutrients on or in the plant tissues occurs and such a competition may also result in the exclusion of less efficient pathogens from the same ecological niche. The efficacy of four strains of *Pseudomonas fluorescens* was assessed for suppressing the development of *Pythium ultimum* infecting pea and promoting the plant growth. All strains of the BCA reduced the number of lesions and the root and soil populations of *Pythium*, while strains SBW25 and CHA0 increased the number of lateral roots of treated plants. The strain SBW25 did not produce any antifungal metabolites and its biocontrol activity was related to its greater colonization ability and rhizosphere competence (Naseby et al. 2001). Application of *Erwinia herbicola* to the blossoms protected the plants against blossom blight phase of fire blight disease caused by *E. amylovora.* Preemptive and competitive colonization of stigmatic surfaces by *E. herbicola* reduced the rate of colonization by *E. amylovora* (Wilson et al. 1992). The efficacy of *Acidovorax avenae* subsp. *avenae* (AAA) (AAA99-2), *Pseudomonas fluorescens* A506 and an unidentified Gram-positive bacterium recovered from watermelon seed (WS-1) was assessed for suppressing infection of watermelon blossom by *A. avenae* subsp. *citrulli* (AAC), causing bacterial fruit blotch (BFB) disease of watermelon. In female blossoms treated with AAC, the pathogen population increased at 96 h post-inoculation. In contrast, pathogen population declined rapidly in blossoms treated with *P. fluorescens* and AAA. Reduction in AAC populations was faster for blossoms treated with *P. fluorescens* strain A506 than for AAA. At 48 h after inoculation, AAC could not be recovered from blossoms treated with either A506 or AAA strains indicating the complete displacement of the pathogen population by the BCA strains (Fessehaie and Walcott 2005).

Antifungal compounds produced by bacterial BCAs have to be delivered in the rhizosphere for effective suppression of soilborne pathogens and this can be

accomplished only by efficient colonization of plant root systems. Identification of root colonization traits will be useful for better understanding of root colonization process. Traits identified as important for root colonization include the motility, the presence of the O antigen of the lipopolysaccharide, synthesis of aminoacids and site-specific recombination. The root colonization gene *sss* which encodes site-specific recombinase has been employed to improve root colonization of wild-type strains of *Pseudomonas* spp. (Dekkers et al. 1998). The efficiency of root colonization by *Pseudomonas fluorescens* WCS 365 and its mutant PCL 1206 was studied. The mutant was 10-to-50-fold impaired in competitive root colonization on tomato, following seedlings inoculation with a 1:1 mixture of parent and mutant strains. The rate of uptake of putrescine by cells of PCL 1206 strain appeared to be increased. Putrescine is an important component of tomato root exudates. It has a bacteriostatic effect on cells of *Pseudomonas* spp. and contains genes involved in putrescine uptake and are highly homologous to the *pot* operon of *Escherichia coli.* A mutation in the promoter region of the *pot* operon resulted in the increased uptake of putrescine which might lead to decreased competitive ability. Hence, the regulated uptake was considered to be an important trait for competition of *Pseudomonas* spp. in the tomato rhizosphere (Kuiper et al. 2001).

The GacS/GacA comprising a two-component regulating system controls the expression of genes required for the synthesis of secondary metabolites with antimicrobial activity in many plant-associated *Pseudomonas* species. Secondary metabolites contributing to biological control that are regulated either directly or indirectly by GacS/GacA system include 2,4-DAPG, phenazines, pyoluteorin, pyrrolnitrin as well as HCN, chitinase and exoproteases. High mutation frequencies of *gacS* and *gacA* in *Pseudomonas* spp. have been observed in liquid culture. In *P. aureofaciens* 30–84, GacS/GacA system controlled the expression of phenazine antibiotics that are inhibitory to pathogenic fungi and enhanced the competitive survival of the BCA. In the natural soil, final rhizosphere populations of wild-type strain 30–84 from mixtures were at least 1.5 times larger than would be predicted from their inoculation ratio and generally were greater than or equal to the population of wild-type alone, despite lower inoculation rates. The results indicated that although *gacS*/*gacA* mutants survive in natural rhizosphere populations, they did not displace wild-type populations (Chancey et al. 2002). *Pseudomonas fluorescens* strain F113 effectively protected beet root plants against *Pythium ultimum*. During colonization of alfalfa rhizosphere, strain F113 produced variants that were characterized by the translucent and diffused colony morphology. The phenotypic variation in this strain appeared to be mediated by the activity of two site-specific recombinases Sss and XerD, since the mutants with disruption in either of the genes involved in the biosynthesis of Sss or XerD showed severe reduction in rhizosphere colonization. Motility is one of the most important traits for competitive rhizosphere colonization and mutants incapable of chemotactic motility were among the most defective colonization mutants. By over-expressing the genes *sss* or *xerD*, a large number of variants (mutants) was generated. By disrupting these genes and complementation analysis, regulation by Gac system of swimming motility by a repression pathway was observed. All isolated variants were more motile than the wild-type strain and

appeared to contain mutations in the *gacA* and/or *gacS* gene. Variants isolated after selection by prolonged cultivation formed a single population with swimming motility that was equal to the motility of *gac* mutants, being 150 % more motile than the wild type. Variants isolated after rhizosphere selection belonged to two different populations: one identical to the population isolated after prolonged cultivation and the other compromising variants that besides a *gac* mutation harbored additional mutations conferring higher motility. The highly motile variants were more competitive than the wild type strain, displacing it from the root tip within 2 weeks (Martinez-Granero et al. 2006).

Colonization of plant roots by introduced PGPR is a complex process involving interactions among the introduced strain, pathogen and native rhizosphere microflora. *Pseudomonas fluorescens* Q8r1-96 is an aggressive colonizer and maintains large populations on the roots of several host plants including wheat, pea, and sugar beet. The role of three genes an *sss* recombinase, *ptsP*, and *orfT* which are important in the interaction of *Pseudomonas* spp. with various hosts was investigated to determine their contribution to the unique colonization ability of the strain Q8r1-96. The *sss* recombinase and *ptsP* genes influenced the global processes, including phenotypic plasticity and organic nitrogen utilization respectively. The *orfT* contributed to the pathogenicity of *P. aeruginosa* in plants and animals and it is conserved among saprophytic rhizosphere pseudomonads. Clones containing the three genes were identified in the genomic library of the strain Q8r1-96. Mutants deficient in these genes were characterized to determine their 2,4-DAPG production, motility, fluorescence, colony morphology, exoprotease and hydrogen cyanide (HCN) production, carbon and nitrogen utilization and ability to colonize rhizosphere of wheat grown in natural soil. The *ptsP* mutant was impaired in wheat root colonization, whereas mutants deficient in the *sss* recombinase and *orfT* were not affected. All the three mutants, however, were less competitive than the parent strain Q8r1-96 in wheat rhizosphere, when they were co-inoculated in pairs with parent strain (Mavrodi et al. 2006).

The mechanism of biocontrol activity of *Pseudomonas fluorescens* isolate 1100–6 effective against *Penicillium expansum* or *P. solitum,* causing apple blue mold was investigated. The wild-type isolate 1100–6 and genetically modified derivative labeled with the gene encoding the green fluorescent protein (GFP) were compared. Both the wild-type and GFP-labeled strain produced large zones of inhibition in dual culture plate tests. Cell-free metabolites of both isolates reduced the colony growth of *Penicillium* isolates to different degrees ranging from 17.3 to 78.5 %. The use of iron chelate did not have a major impact on the antagonistic activity of *P. fluorescens.* The wild-type and GFP-labeled isolates significantly reduced the incidence and severity of apple at 11 days after inoculation at 20 °C and by *P. expansum* and *P. solitum* after 25 days at 5 °C, when the BCA isolates were applied in wounds 24 or 48 h before challenge inoculation with the pathogens. The population of labeled isolate increased in the wound from log 6.95 at the time of inoculation to log 9.12 CFU/ml at 25 days after inoculation at 5 °C. The results indicated that the BCA suppressed the blue mold pathogen through antagonism by producing toxic metabolites and it did not penetrate deep into the wounds made on apples (Etebarian et al. 2005).

Application of genetically modified microorganisms (GMMs) is suggested as an approach to overcome the problem of inconsistency in the performance of biocontrol agents against crop pathogens. The effects of introduced wild-type strains and GMMs on the soil ecosystem have been studied in certain microcosms. The impact of genetically modified, antibiotic-producing *Pseudomonas putida* WCS58r and its two transgenic derivatives was investigated by introducing them as a seed coating into the rhizosphere of wheat in two consecutive years (1999 and 2000) in the same field plots. The transgenic derivatives WCS 358r :: phz and WCS358r :: phl constitutively produced phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (DAPGs) respectively. The *phz* and *phl* genes stably persisted in the chromosomes of the respective derivatives. The amount of PCA produced in the wheat rhizosphere by the transformant was about 40 ng/g of roots after the first application in 1999. The DAPG-producing derivatives caused a transient shift in the indigenous bacterial and fungal flora in the first year, as determined by amplified ribosomal DNA restriction analysis (ARDRA). However, after the second application of the transformant in 2000, the bacterial or fungal flora was not altered. All bacterial treatments showed positive effect on plant growth, possibly due to suppression of microbial plant pathogens. The results suggested that the effects of GMMs on microflora might be within the range of natural variability as induced by agricultural practices like crop rotation (Viebahn et al. 2003).

5.1.1.3 Prevention of Pathogen Colonization by Bacterial Biocontrol Agents

Suppression of the pathogen and disease development may be achieved, when a biocontrol agent is able to prevent colonization of specific host tissues by the pathogen. *Rhizobium (Agrobacterium) vitis* causes crown gall disease in grapevines. *Pseudomonas fluorescens* (*Pf*) isolate 1100–6 showed potential for suppressing the formation of galls. *Nicotiana glauca* plants were used to study the impact of the BCA, because large uniform gall developed, following inoculation with *R. vitis.* Prophylactic treatment of *N. glauca* with the *Pf* isolate 1100–6 reduced drastically the gall size produced by *R. vitis* and disease suppressive effect was seen at least 86 days after the BCA was applied. The results indicated that the isolate 1100–6 interfered with gall initiation, even when the plants were challenged with *R. vitis* only 20 min after treatment with BCA isolate. The kinetics of inhibition of gall formation by Pf isolate 1100–6 could be the result of direct physical association of the BCA with *R. vitis* or to the plant cells at the wound sites, providing a physical barrier and direct interference with the adhesion process which is an essential requirement for plant cell transformation. Visualization of *Pf* isolate 1100–6 *gfp* (tagged with green fluorescent protein) by epifluorescent microscopy confirmed the ability of this isolate to propagate in the internal and external surfaces of grapevine. Colonization of xylem vessels and pith tissues of grapevines by *Pf* isolate 1100–6 provided evidence for the suitability of this isolate to be applied on grapevines. In addition, the *Pf* isolate 1100–6 was able to survive on the rhizosplane of grapevines for 6 months (Eastwell et al. 2006).

Application of *Pseudomonas fluorescens* CH31 to cucumber significantly reduced root colonization by *Pythium aphanidermatum* and consequently root rot disease development was significantly suppressed (Moulin et al. 1996). Treatment of sugar beet seeds with *P. putida* 40 RNF as pellets significantly reduced the incidence of sugar beet damping-off disease caused by *Pythium ultimum*. The bacterial BCA reduced seed pericarp colonization by *P. ultimum* by 43 % at 48 h after planting and caused a reduction in the number of sporangia produced by 68 % in the soil around the plants. Treatment with *P. putida* was equally effective as the fungicide hymexazole in reducing disease incidence in infested soils (Shah-Smith and Burns 1996). A competitive root tip colonization assay was developed to select the efficient root colonizers of tomato root tips from the bacterial isolates obtained from tomato rhizosphere soil samples. Based on this trait 24 isolates that were equally or better colonizing ability relative to the well known efficient root colonizer *Pseudomonas fluorescens* WCS365. This procedure facilitated the isolation of seven new biocontrol strains including *Pseudomonas rhodesia* effective against tomato foot and root rot (TFRR) disease caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Validov et al. 2007).

The stigma of rosaceous plants being apparently nonselective supports the growth of microorganisms that are pathogenic as well as commensal epiphytes, some of which are antagonistic to *Erwinia amylovora,* causative agent of fire blight disease. Avirulent *hrp* regulatory mutants were found to partially protect greenhouse-grown seedlings from shoot and blossom infection by *E. amylovora* (Tharaud et al. 1997; Faize et al. 2006). The antagonistic bacteria, after establishing themselves on stigmas, utilize the mechanism of competitive exclusion and/or antibiosis to suppress pathogen development. When virulent *E. amylovora* strain Ea153N was inoculated individually on stigmas of pear, apple and black berry, it reached the highest population levels which were double that of the avirulent *hrpL* mutant of Ea153N or bacterial antagonists *P. fluorescens* and *Pantoea agglomerans*. In competition experiments, growth of the avirulent derivative was suppressed by the antagonist mixture to a greater extent than the virulent strain. Addition of a small amount of virulent Ea153N strain to the inoculum of an avirulent *hrpL* mutant significantly increased the population of the avirulent strain. In the orchard trials (9), the avirulent *hrpL* strain significantly suppressed the incidence of fire blight in four trials, whereas the antagonist mixture reduced the disease incidence significantly in six trials (Johnson et al. 2009).

Rhizosphere colonization by biocontrol agents depends on several factors, of which motility is an important trait. In *Pseudomonas fluorescens* F113 rif (F113), motility is a polygenic trait that is repressed by at least three independent pathways. The *kinB* gene encoding a signal transduction protein that together with *AlgB* was implicated in alginate production, participated in swimming motility repression through the Gac pathway, acting downstream of the GacAS two-component system. The Gac mutants were found to be impaired in the production of secondary metabolites, resulting in the loss of biocontrol activity of the mutants. But the *kinB* mutant and a triple mutant impaired in kinB, *sadB* and *wspR* (KSW) had a wild-type phenotype for secondary metabolism. The strain KSW was found to be hypermotile and more

competitive for rhizosphere colonization than the wild-type strain. The biocontrol potential of the mutants was compared with the wild-type strain. The strain KSW possessed improved biocontrol efficiency, compared with wild-type strain, indicating biocontrol potential of the strain could be enhanced by increasing its competitive colonization ability (Barahona et al. 2011).

5.1.1.4 Targeting Pathogenic Potential

Plant pathogens produce different kinds of toxins which may be host-specific or host-nonspecific (general). The host-specific toxins (pathotoxins) are able to induce the major symptoms of the disease as the pathogen itself, whereas host non-specific toxins do not have a major role in symptom development. Some fungal pathogens like *Fusarium culmorum* produce mycotoxins that are harmful, to animals and humans, when contaminated food and feed are consumed. *F. culmorum* causes seedling blight of wheat and barley. This pathogen produces trichothecene and synthesis of this mycotoxin is governed by the gene encoding the trichodiene synthesis. The BCAs *Pseudomonas fluorescens* strains MKB 100 and MKB249, *P. frederiksbergensis* strain 202 and *Pseudomonas* sp. MKB 158 were able to reduce significantly the extent of coleoptile growth retardation and seedling blight disease incidence. These bacterial strains were effective in ameliorating the negative effects of *F. culmorum* on seedling germination. The *Tri5* gene encodes trichodiene synthase that catalyzes the first step in the trichothecene mycotoxin biosynthetic pathway in *Fusarium* spp. Expression of *Tri5* gene was reduced by 33 % in stem base tissue coinoculated with *Pseudomonas* sp. strain MKB 158. As coinoculation of *Pseudomonas* sp. strain MKB 158 induced the expression of a wheat class III plant peroxidase gene, the biocontrol activity of the bacterial strain might be due to induction of systemic resistance to seedling blight disease in wheat (Khan et al. 2006).

5.1.1.5 Induction of Resistance to Crop Diseases

Resistance to crop diseases may be induced in plants by applying various biotic and abiotic agents. Induced resistance may be broadly differentiated into two forms as systemic acquired resistance (SAR) and induced systemic resistance (ISR). In response to pathogen infection or chemical application, “SAR” process is initiated, whereas colonization of plant roots by PGPR leads to ISR. SAR is considered to be activated more commonly by the pathogens, causing cell death reactions, ranging from single-cell hypersensitive reaction [(HR), a plant-specific type of programmed cell death (PCD)] to necrotic disease lesions. SAR is mediated by a salicylic acid (SA)-dependent process (Gaffney et al. 1993). On the other hand, ISR is mediated by jasmonate-or ethylene-sensitive pathway (Pieterse et al. 1998; Walters et al. 2005). SAR is characterized by an early increase in endogenously synthesized SA, coupled with the concomitantly activation of a set of SAR genes (Ryals et al. 1996; Sticher et al. 1997). ISR elicited by PGPR is capable of suppressing disease

development in aerial plant organs which are far separated from the roots that are treated with BCA in various plant species (van Loon et al. 1998; Kloepper et al. 2004).

When a plant is appropriately stimulated, a state of enhanced defensive ability known as induced resistance is observed in the plant. Induced systemic resistance (ISR), as the mode of action of disease suppression by nonpathogenic bacteria present in the rhizosphere of plants, was suggested by two groups of researchers working independently (Van Peer et al. 1991; Wei et al. 1991). SAR requires accumulation of salicylic acid (SA) in the model plant *Arabidopsis thaliana*. ISR is dependent on intact responses to ethylene and jasmonic acid. When these two different signal transduction pathways were triggered simultaneously in *A. thaliana,* the effectiveness of disease suppression was significantly enhanced (van Wees et al. 2000). The bacterial determinants involved in induction of systemic resistance in different host plants by bacterial biological control agents are presented in Table 5.5. The bacterial metabolite SA itself was suggested to trigger the SA-dependent signal transduction pathway. *Pseudomonas fluorescens* strain WCS 374 produced relatively large quantities of SA under iron-limited conditions, compared with conditions of sufficient iron availability. However, no indication for activation of SA-dependent signaling in radish after WCS374 treatment could be noted. WCS 374 strain was more effective in radish against Fusarium wilt under iron-limited conditions (Leeman et al. 1995). The iron- regulated ISR was considered to be the mechanism of biocontrol activity by WCS 374 in radish through mediation by the SA-containing siderophore pseudomine produced by the strain WCS37 (Mercado-Blanco et al. 2001). In the case of *P. aeruginosa* strain 7NSK2 with ability to produce SA, evidence suggested that SA was not the agent inducing resistance, but the compounds pyochelin and pyocyanin produced by this strain were the prerequisite for ISR induced by 7NSK2 strain. Thus SA produced by bacterial strains did not appear to be involved directly in the ISR against plant diseases (Audenaert et al. 2002).

Salicylic acid (SA)-induced defense expression via nonexpressor of pathogenesis related protein (PR)-genes-1 (*NPR1*), a key mediator present in plants. *NPR1* encodes a novel protein with a bipartite nuclear localization sequence and two potential protein-protein interaction domains (Cao et al. 1997; Dong 2004). Activity of NPR1 is dependent on the cellular redox. In *Arabidopsis*, AtNPR1 is predominantly in a monomeric form that can translocate into the nucleus, where it activates defense gene expression through interaction with transcription factors (Subramaniam and Desveaux 2001; Zhang et al. 2003). *Pseudomonas fluorescens* induces ISR which is also mediated by NPR1 as SAR (Spoel et al. 2003). Some strains of *Bacillus* spp. induced ISR dependent on SA and independent of jasmonic acid (JA) and *NPR1*. Furthermore, ISR induced by *Pseudomonas* spp. did not result in accumulation of PR1 in plants. On the other hand, ISR induced by *Bacillus* spp. in some plants, led to accumulation of PR1 in treated plants (Kloepper et al. 2004). *Pseudomonas aeruginosa* 7NSK2 produced SA, a siderophore, that was found to be primarily responsible for induction of ISR to *Botrytis cinerea* causing gray mold disease of bean and this resistance was shown to be iron-regulated (Meyer and Höfte 1997). Root inoculation of *Arabidopsis thaliana* with *P. fluorescens* CHA0 resulted in partial protection

Table 5.5 Bacterial determinants of eliciting systemic resistance in plants against microbial plant pathogens

Bacterial species/strains	Plant host/nature of bacterial determinant	References
Bacillus spp.	2-aminobenzoic acid (2-AB)	Yang et al. (2010)
B. cereus	Tomato/cell dialysates containing macromolecules	Romeiro et al. (2005)
Bacillus mycoides Bac J	Sugar beet/PO, chitinase and β-1,3-glucanase	Bargabus et al. (2002)
B. pumilus	Sugar beet/PO, chitinase, β-1,3-glucanase	Bargabus et al. (2004)
Bacillus subtilis S499	Potato/surfactin	Ongena et al. (2007)
B. subtilis GBO3 and IN 937a	*Arabidopsis* – 2,3 butanediol	Ryu et al. (2004)
Chrysobacterium balustinum AUR 9	*A. thaliana* colo lipopolysaccharides	Solano et al. (2008)
Pseudomonas aeruginosa 7NSK2	Pyochelin and pyocyanin Cotton/ siderophore – pseudobactin	Audenaert et al. (2002), Meyer and Höfte (1997), and Fallahzadeh – Mamaghani et al. (2009)
P. chlororaphis 06	Tobacco/2R, 3R- butanediol	Han et al. (2006)
P. fluorescens CHA0	Tobacco/siderophore	Maurhofer et al. (1994)
P. fluorescens WCS 374	*Arabidopsis thaliana*/salicylic acid-containing siderophore	Leeman et al. (1995)
	Radish/lipopolysaccharide/ siderophore	Mercado-Blanco et al. (2001)
P. fluorescens WCS417	Carnation/lipopolysaccharide	Van Peer and Schippers (1992)
P. putida BTP1	Bean/N-alkylated benzylamine derivative (NABD)	Ongena et al. (2004)
Paenibacillus spp. B2	*Medicago truncatula*/Paenimyxin (lipopolypeptide)	Selin et al. (2010)
Serratia marcescens 90-166	Siderophore – catechol	Press et al. (2001)

of leaves against *Peronospora parasitica* causing downy mildew disease. Induction of ISR to *P. parasitica* required the synthesis of the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) by *P. fluorescens*, as application of 2,4-DAPG at 10–100 μM mimicked the ISR effect, indicating the possibility of similar mechanism operating in other pathosystems also (Iavicoli et al. 2003). Another investigation using several strains of *Pseudomonas* spp. indicated that elicitation of ISR was typically dependent on SA and did not result in activation of *PR-1a* gene that encodes PR-1a protein (van Loon and Glick 2004). *P. putida* BTP1 applied on the roots of bean reduced the symptoms of gray mold disease caused by *Botrytis cinerea* on leaves. The molecular determinant of *P. putida* involved in ISR was isolated from cell-culture fluid after growth. The N-alkylated benzylamine derivative (NABD) purified from culture

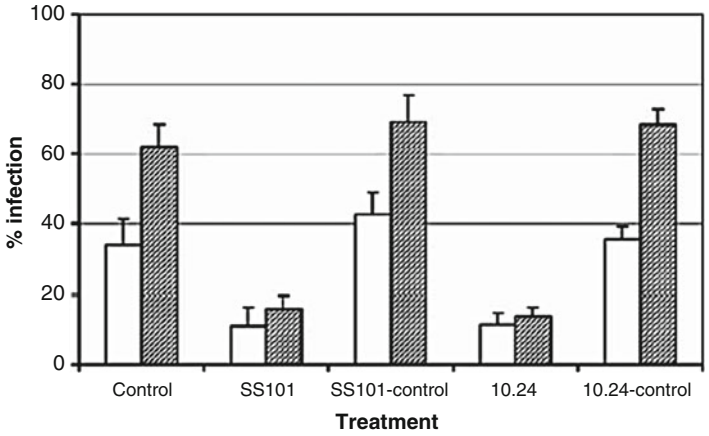

Fig. 5.4 Effect of treatment of wheat or apple with *Pseudomonas fluorescens* SS101 or *massA* mutant 10.24 on root infection by *Pythium* spp. assessed by split-root assays. *White bars*: infection of wheat; *gray bars*: infection of apple. Infection rates for SS101 and 10.24 treatments were significantly lower than the control treatments ($P < 0.001$) (Courtesy of Mazzola et al. 2007 and with kind permission of The American Phytopathological Society, MN, USA)

fluid mimicked the protective effect as induced by crude samples. The mutants impaired in NABD biosynthesis lost the elicitor activity, indicating the requirement of NABD for ISR induction (Ongena et al. 2004).

A split-root plant assay was applied to determine, whether suppression by *Pseudomonas fluorescens* SS101 of *Pythium* spp. pathogenic to wheat or apple, was due to direct or indirect effects. The assays were conducted in orchard soils to explore the possible role of induced resistance. Strain SS101 or the *massA* mutant 10.24 significantly reduced infection by *Pythium* spp. on the component of the apple or wheat root system cultivated in soil treated with the respective bacteria. Wheat root infection was reduced from ≈34 % for plants grown in non-treated soil to ≈11 % for the component of plant root systems grown in SS101- or mutant 10.24-treated soils. Infection of 'Gala' apple seedling roots was reduced from 60 to 70 % in the control to ≈15 % for the portion of the root mass cultivated in SS101- or 10.24-treated soils. For the SS101 and 10.24 treatments, no difference in frequency of *Pythium* root infection was observed for the component of the root system physically separated from bacterially treated soil, relative to that observed for control treatment (Fig. 5.4) (Mazzola et al. 2007).

The capacity of strains of *Pseudomonas* spp. to induce systemic resistance in *Ecalyptus urophylla* against bacterial wilt disease caused by *Ralstonia solanacearum* was assessed. Two strains *P. putida* WCS 358r and *P. fluorescens* WCS374r could trigger ISR, when infiltrated into two lower leaves at 3–7 days before challenge inoculation, but not when these strains were applied to the soil. A mutant of strain WCS 358r defective in the biosynthesis of the siderophore pseudobactin did not induce ISR. The purified siderophore from WCS358r did induce ISR, suggesting

that pseudobactin 358 was the ISR determinant of WCS358r strain. A siderophore-minus mutant of WCS374r could induce disease resistance to the same level as the wild-type strain. The purified siderophore from this strain could induce ISR, indicating that both the siderophore and other uncharacterized ISR determinant of WCS 374r could trigger ISR in *Eucalyptus.* Although soil drench with salicylic acid (SA) induced ISR in *Eucalyptus,* transformation of a siderophore-minus mutant of WCS358 with SA biosynthetic gene cluster from WCS 374 did not restore the ability to cause ISR in *E. urophylla* (Ran et al. 2005). Many determinants of ISR such as iron-regulated metabolites such as pseudobactin siderophore have been demonstrated to have an important role in ISR (Meziane et al. 2005). Production of the siderophores relies on the concentration of iron in the environment and under conditions of low iron availability. Purified siderophores have disease suppressive effect similar to that of the strain producing the siderophore (Neilands and Leong 1986). King's medium B supplemented with 8-hydroxyquinoline (8-HQ) (120 ppm), a chelator, was used for screening of *Pseudomonas* spp. from the rhizosphere of cotton, in order to select the isolates with capacity to produce high concentrations of siderophores that might be equal to or greater than the reference strain *P. aeruginosa* 7NSK2. The strains 35Q and 16Q produced significantly greater quantities of siderophore, the strain 35Q producing a maximum of 381.7 μM. In hydroponic experiments with cotton, the bacterized plants with low iron availability showed high levels of resistance to cotton bacterial blight disease caused by *Xanthomonas campestris* pv. *malvacearum* (*Xcm*). At 48 h after inoculation of plants with *Xcm*, the levels of defense-related enzymes peroxidase (PO) and phenylalanine ammonia lyase (PAL) were significantly higher in plants treated with the strain 35Q. The strain 35Q was found to be a powerful producer of pseudobactin and this siderophore might be a determinant of induction of systemic resistance in cotton under iron-limited conditions (Fallahzadeh-Mamaghani et al. 2009).

The biocontrol potential of grapevine-associated bacterial species *Pseudomonas fluorescens* PTA-CT2, *Acinobacter lwoffii* PTA-113 and *Pantoea agglomerans* PTA-AF1 was assessed against *Botrytis cinerea*, causative agent of gray mold disease in two vineyards with susceptible cv. Chardonnay. The severity of the disease on grapevine leaves and berries was reduced to different levels, depending on the bacterial strain and inoculation method. Systemic resistance was stimulated in grapevine plants drenched with these bacterial BCAs, with a single application. The ISR was associated with a stimulation of plant defense responses such as chitinase and β-1,3-glucanase reaching its maximum activity at ripening stage. The enzyme activities reached their maximum in leaves of grapevine plants treated with *P. fluorescens* and *A. lwoffii*, whereas the peaks of enzyme activities in the berries of plants treated with *A. lwoffi* and *P. agglomerans* were detected. *A. lwoffii* was able to stimulate the activities of chitinase and β-1,3-glucanase more efficiently in both leaves and berries which are vulnerable to the infection by *B. cinerea* (Magnin-Robert et al. 2007). The biotic inducers, *Pseudomonas fluorescens* and *P. putida* were evaluated for their efficacy in inducing systemic resistance in lupine against Fusarium wilt disease caused by *F. oxysporum* f.sp. *lupine* (FOL), when applied as seed treatment. Both biotic inducers significantly reduced the disease incidence under greenhouse

and field conditions. *P. fluorescens* and the abiotic inducer potassium chloride were more effective than *P. putida*, copper sulfate and indole butyric acid. A time-course of defense-related enzymes showed substantial increases in enzymatic activities in induced infected seedlings, compared with untreated unhealthy plants or infected controls. The extent of increase in enzymatic activities varied among treatments. Maximum increases in chitinase and β-glucanase activities were recorded at 12 and 18 days after inoculation with the pathogen respectively. In addition, phenylalanine ammonia lyase (PAL) increased substantially at 8 days after inoculation. Phenolic compounds and specific flavonoids accumulated markedly following infection by FOL in induced and/or infected seedlings compared with healthy plants. The inducers increased the crop parameters and seed yield, compared with untreated control plants (Abd El-Rahman et al. 2012).

Pseudomonas chlororaphis induced ISR in tobacco and cucumber plants against two bacterial pathogens *P. syringae* pv. *tabaci* and *Erwinia carotovora* sub sp. *carotovora* (Spencer et al. 2003) as well as against the fungal pathogen *Corynespora cassiicola* causing leaf spot disease (Kim et al. 2004a, b). Investigations were carried out to monitor the transcriptional response of over 8,000 *Arabidopsis* genes in order to identify ISR-related genes. *P. chlororaphis* 06-mediated ISR was associated with rapid induction of several genes after a challenge inoculation with *C. cassiicola* in cucumber leaves relative to the controls treated with water (Kim et al. 2004a, b). After challenge inoculation with *P. syringae* pv. *tomato* on the *P. fluorescens* WCS 417r-induced plants, 81 genes were evidenced augmented expression patterns within the leaves. These genes were primed to respond faster or more strongly, when exposed to invasion by pathogens. The capacity for augmented defense expression is designated "priming". This process of priming has been observed in several plant species protected by ISR induced by bacterial biocontrol agents (Verhagen et al. 2004).

Induced systemic resistance (ISR) elicited by PGPR has been demonstrated in several crops including bean, carnation, cucumber, radish, tobacco, tomato and *Arabidopsis thaliana* (Van Loon et al. 1998). Plant defensive systemic responses induced by three PGPRs *Azospirillum brasilense* Sp7, *Chrysobacterium balustinum* AUR9, and *Pseudomonas fluorescens* AUR6 on *Arabidopsis thaliana* Col O against *P. syringae* pv. *tomato* (Pst) DC 3000 were studied at the biochemical and transcriptional levels. All three bacterial strains reduced disease severity, when applied prior to challenge inoculation with *Pst.* The maximum protection against the disease was provided by *C. balustinum*. Plants treated with each of the three strains were also reduced in salicylic acid (SA) production after pathogen challenge, compared to untreated controls. *C. balustinum* AUR induced maximum production of SA. The expression level of pathogenesis-related protein PR-1, a transcriptional marker of SA-dependent pathway in *C. balustinum* AUR9-treated plants, was four-fold that of controls, whereas the expression of PDF1.2, a transcriptional marker for the SA-independent pathway was not induced. SA production by PGPR strains could be attributed to the effect of PGPR strains identified as avirulent pathogens. The protection conferred was inversely related to SA production and coincided with reduction of SA accumulation in PGPR-treated plants. *C. balustinum* cell wall lipopolysaccharides being putative bacterial elicitor molecules, were able to

reproduce the systemic induction effect at low doses. It is possible that some PGPR strains may stimulate different systemic responses in host plants. With *C. balustinum* AUR9, the SA-dependent pathway was stimulated first, as indicated by increases in SA levels and PR1 expression, followed by induction of the SA-independent pathway, as indicated by the increases in ethylene (ET) concentrations. The role of ET on ISR-mediated response was detected by its sensitivity (priming response). The PGPR strains giving higher levels of protection against the pathogen caused the release of greater amounts of ET in plants after incubation with its precursor 1-amino-cyclopropane-1-carboxylic acid (ACC). These strains also blocked the SA peak upon pathogen challenge. The induction of both pathways appeared to result in additive effect on disease suppression (Solano et al. 2008).

The impact of strains of *Pseudomonas fluorescens* capable of inhibiting soilborne fungal pathogens during colonization of the wheat rhizosphere was studied. Based on the assumption that *P. fluorescens* induced defense genes in wheat roots, a microarray 192 oligonucleotides representing 84 wheat root expressed sequence tags (ESTs) homologous to defense stress genes were constructed. The ESTs were selected from the wheat EST libraries. Four days after seed inoculation with wheat take-all suppressive strain *P. fluorescens* Q8r1-096, the arrays were integrated with labeled transcript (cDNA) populations from roots or coleoptiles of the cv. Finley. The transcripts encoding jasmonic acid pathways and proteins associated with the hypersensitive response, in addition to stress associated proteins, were induced or repressed in wheat roots during *P. fluorescens* interactions. Transcripts encoding PR-protein Pr-10a and hypersensitive response protein HR in 1 h were also induced in coleoptiles. Real-time PCR assay showed that 11 transcripts were induced in root tissues between 2 and 6 h and remained at higher levels at 24 h post-inoculation. The results suggested that defense/stress gene expression might be modulated by *P. fluorescens* in wheat root tissues (Okubara et al. 2010). The biocontrol mechanism of *Pseudomonas chlororaphis* MA342 and/or *Serratia plymuthica* HRO-C489 applied as seed treatment against *Verticillium longisporum* infecting oilseed rape was investigated. Soil was infested with microsclerotia and mycelium of *V. longisporum* followed by planting seeds treated with rifampicin-resistant bacterial strains at the rate of 10^{6-7} cells/seed. A significant reduction in disease intensity, as reflected by values of area under disease progress curve (AUDPC) was recorded for both bacterial strains. Significant differences in the percentage of healthy plants were noted between the cultivars ranging from 46.5 to 72.6 %. No additional benefit was evident due to combined application of the BCA strains. Growth promotion effects due to the application of BCAs were not related to the extent of disease control. As the possibility of containing this disease through cultivar resistance and/or chemical application is remote, the use of the PGPR that can raise the level of resistance to the disease has to be comprehensively examined (Abuamsha et al. 2011).

Various strains of rhizobacteria have been reported to suppress the development of several plant diseases through induction of resistance in the host plant species. Induced systemic resistance is mediated mainly by fluorescent *Pseudomonas* spp. and also by endophytic bacteria such as *Bacillus* spp. ISR induced against *Tobacco necrosis virus* by *P. fluorescens* CHA0 coupled with stimulation of PR

protein synthesis in tobacco was demonstrated by Maurhofer et al. (1994). Stimulation of phytoalexin synthesis following PGPR treatment was observed in carnation treated with *Pseudomonas* sp. (Van Peer et al. 1991). In a later study, cucumber plants were protected against *Pythium aphanidermatum* causing root rot disease by application of *P. putida* isolate BTP1 and its sid^- mutant M3. The protection was primarily associated with accumulation of antifungal compounds (phenolics) in treated roots. The phenolics were the phytoalexins produced systemically. Analyses of leaf samples revealed increased concentrations of fungitoxic molecules in PGPR-treated plants, although the nature of these molecules appeared to be different from those detected in roots. The results suggested that the PGPR might elicit phytoalexins systemically in cucumber and the overall defense response was not based on a single phytoalexin, but it is chemically complex and organ-specific (Ongena et al. 2000). Watermelon in Mekong Delta of Vietnam was affected seriously by *Didymella bryoniae,* the causative agent of gummy stem blight disease. *Pseudomonas aeruginosa* strain $23_{1\text{-}1}$ suppressed the development of *D. bryoniae* directly by the production of antibiotics locally and/or indirectly by stimulating the defense systems systemically. Foliar infection by *D. bryoniae* was significantly reduced by treating the seeds with *P. aeruginosa,* indicating the ability of the bacteria to induce ISR in watermelon under field conditions. *P. aeruginosa* colonized the watermelon plants endophytically, more actively in infected plants than in healthy plants. Treatment of watermelon seeds with BCA inhibited pathogen penetration which was associated with accumulation of H_2O_2, followed by enhanced peroxidase activity and occurrence of new peroxidase isoforms. The mechanisms of biocontrol activity of *P. aeruginosa* against *D. bryoniae* were antibiosis and ISR under greenhouse and field conditions. The PGPRs present in the native soils have to be screened for their biocontrol potential to select and apply the most efficient strains capable of surviving under field conditions (Nga et al. 2010).

The potential of *Pseudomonas fluorescens* 89B61 to induce ISR in tomato against the late blight pathogen *Phytophthora infestans* was evaluated. This strain elicited systemic protection against the disease and reduced disease severity by a level equivalent to SAR induced by the chemical β-amino butyric acid (BABA) in greenhouse experiments. The results suggested that protection induced by *P. fluorescens* was SA-independent, but ethylene- and jasmonic acid-dependent, whereas SAR elicited by BABA was SA-dependent. Further, the lack of colonization of tomato leaves by strain 89B61 suggested that the ISR was due to systemic protection by this strain and not attributable to a direct interaction between the pathogen and the BCA (Yan et al. 2002). The bacterial strains with multiple mechanisms for biocontrol activity against fungal pathogens are likely to be more efficient. *P. chlororaphis* strain PA-23 produced phenazine-1-carboxylic acid (PCA) and acetamidoanthranilic phenol (AAP) which inhibited the mycelial growth of *Sclerotinia sclerotiorum,* causing stem rot disease of canola. The germination of ascospores of *S. sclerotiorum* in canola petals was inhibited by the strain PA-23, as revealed by microscopic observations. Two applications of PA-23 induced resistance against infection by *S. sclerotiorum*. Enhanced accumulation of PR proteins and oxidative enzymes including chitinase and β-1,3-glucanase by PA-23 in canola leaf tissues

might account for reduction in pathogen infection. The combination of antibiotic production with induction of ISR by *P. chlororaphis* PA-23 could act synergistically in restricting the pathogen growth and colonization, resulting in significant suppression of disease incidence and intensity (Fernando et al. 2007).

Systemic resistance against plant pathogens induced in plants by biocontrol agents and chemicals has been investigated to have an insight into the variations in the biochemical and molecular genetic bases between normal healthy and treated plants, following challenge inoculation with the microbial pathogens. Ultra-structural changes in treated plants resulting in resistance to invading pathogens have been studied only in a few cases. Infection behavior of *Colletotrichum orbiculare,* causing cucumber anthracnose disease, was monitored in the leaves of cucumber preinoculated with *Pseudomonas fluorescens* 89B61, *Serratia marcescens* 90–166, using transmission electron microscope (TEM). Active defense responses such as sheath formation at penetration sites and accumulation of endoplasmic reticula or numerous vesicles around intracellular hyphae were observed in the leaves of plants preinoculated with both bacterial strains. In addition, the electron densities of most intracellular and intercellular hyphae were greater than those of untreated control plants which did not exhibit any of the defense responses detected in BCA-treated leaves as indicated above. In plants treated with chemical inducers of disease resistance like DL-3-amino butyric acid or aminosalicylic acid, no active defense responses were observed and the pathogen hyphae were rarely seen at 5 days after challenge inoculation. The results suggested that the mechanisms of ISR were differentially expressed in plants treated with bacterial BCAs and chemical inducers (Jeun et al. 2007).

The antagonistic activity of *Pseudomonas syringae* against fungal pathogen, causing citrus postharvest diseases was correlated with in vitro production of lipodepsipeptides and induction of resistance in citrus tissues as well, due to a broad spectrum of metabolic modifications. The expression of syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes from *P. syringae* pv. *syringae* (*Pss*) biocontrol strains was assessed on different culture media and in vivo on citrus fruits during interaction with *Penicillium digitatum* by quantitative RT-PCR assay. The *syrB1* and *sypA* genes were more actively expressed, when *Pss* strains were grown on orange peel broth, as compared to nutrient broth and potato dextrose broth. Infection by *P. digitatum* was strongly stimulatory only to *syrB1* expression, suggesting that *syrB1* gene could be involved in the biocontrol activity. Results of QRT-PCR indicated that both *Pss* and *P. digitatum* could enhance transcription of CHI1 in inoculated flavado tissues, compared with untreated control. Further, coinoculation of *Pss* and the pathogen strongly induced the transcription of CHI1. The CHI1 gene was considered to be the most possible part of molecular mechanisms involved in the pathogen defense responses in citrus fruit (Scuderi et al. 2011).

The mechanism of biocontrol activity of endophytic *Pseudomonas putida* MGY2 isolated from papaya fruit against the anthracnose disease of papaya caused by *Colletotrichum gloeosporioides* was investigated. Treatment with MGY2 significantly reduced disease incidence and lesion diameter in papaya fruit inoculated with *C. gloeosporioides*. The strain MGY2 was found to inhibit ethylene production and

reduced the decline of firmness of harvested papaya fruits stored at 25 °C, resulting in maintenance of natural resistance in papaya fruits. MGY2-treated papaya fruit maintained a significantly higher level of PAL activity from day 3 to day 9 of incubation compared to the control fruit. The BCA treatment also enhanced the total phenol contents of the fruits. During the storage, the levels of phenolics in MGY2-treated fruits were higher than the untreated controls. The bacterial treatment induced catalase (CAT) and peroxidase (PO) activities to reach levels significantly higher than in untreated fruits. CAT and PO are important detoxifying enzymes which are known to function together with other enzymes in the ascorbate glutathione cycle for promoting the scavenging of reactive oxygen species (ROS). The expressions of PAL1, CAT1 and PO were analyzed by northern blotting. The expression of PAL1 in MGY2-treated fruit appeared to be stronger compared to controls, as reflected by the mRNA levels which were elevated in MGY2-treated fruits. The reduction of decay in papaya fruit treated with MGY2 might be, due to a combined action of the defense-related enzymes which were stimulated significantly following application of the bacterial strain (Shi et al. 2011) (Appendix 5.4).

Pseudomonas chlororaphis 06, has been reported to induce resistance in tobacco against soft rot pathogen *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *P. syringae* pv. *tabaci* (*Pst*) causing wild fire disease. The extracellular compounds produced by *P. chlororaphis* were purified to identify the bacterial determinants involved in induction of disease resistance. Based on the results of high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) mass spectrophotometry, the active compound was identified as 2R, 3R-butanediol. This compound induced systemic resistance to *Ecc* SCC1, but not to *Pst*. Treatment of tobacco with volatile 2R, 3R butanediol enhanced aerial growth which was also observed during colonization by *P. chlororaphis*. The global sensor kinase GacS of *P. chlororaphis* 06 was found to be a key regulator for ISR against *Ecc* through regulation of 2R, 3R-butanediol production. The investigation seems to be the first in linking the ISR to 2R, 3R-butanediol, a fermentation product as well as to the sensor kinase GacS for its production (Han et al. 2006). The mechanism of biocontrol potential of *Pseudomonas fluorescens* BK3 against the apple fire blight pathogen *Erwinia amylovora* was studied. The susceptible cultivar Holsteiner Cox was pretreated with 1×10^6 cells of the strain BK3 at 2 days prior to challenge inoculation with *E. amylovora.* The intracellular washing fluid (IWF) was collected from the plants before and after treatment with the BK3. The IWF from treated plants contained chitinase, β-1,3-glucanase, thaumatin-like protein and ribonuclease which belonged to the group of pathogenesis-related (PR) proteins. Investigations on transcript level revealed the up-regulation of 113 EST clones which also belonged to the class of PR-proteins, oxidative stress and transcripts that code for proteins which play important role at different stages of pathogen recognition and signaling pathways (Kürkcüoglu et al. 2007). A noninvasive method of determining populations of BCA and pathogens on the leaf surface or inside plant tissues based on bioluminescence was developed. The pathogen *E. amylovora* and *P. fluorescens* BK3 were transformed with *lux* CDABE gene cluster that codes for two structural genes of the luciferase as well as

for the genes of the substrate biosynthesis. The measurement of bioluminescence revealed that the strain BK3 protected the apple plants, when treated at 2 days prior to inoculation with *E. amylovora.* Application of the strain BK3 did not cause any visible morphological change in treated plants. The results indicated that the strain BK3 protected the apple plants by inducing resistance to fire blight disease (Schmoock et al. 2008).

Pseudomonas fluorescens strains have been reported to induce systemic resistance to some virus diseases affecting different crops. *P. fluorescens* strain 89B-27 induced systemic resistance to *Cucumber mosaic virus* (CMV) in cucumber cv. Straight 8 leading to consistent reduction in the mean numbers of symptomatic plants coupled with delay in symptom expression. No viral antigen could be detected in the asymptomatic plants throughout the experimental period (Raupach et al. 1996). The strains Pf1 and CHA0 of *P. fluorescens* were able to induce systemic resistance in rice against tungro disease, when these BCA strains were applied as seed treatment, root dipping or foliar spray (Narayanasamy 1995). Enhancement of the activities of defense-related enzymes such as peroxidase (PO) and phenylalanine ammonia lyase (PAL) was observed in many crop plants treated with the PGPR strains (Narayanasamy 2005). The ability of three strains CoP-1, CoT-1 and CHA0 of *P. fluorescens* to induce ISR in tomato against *Tomato spotted wilt virus* (TSWV). The BCA strains were used to treat the seed, soil, roots of seedlings and foliage of transplanted tomato plants. Treatment with BCA strains resulted in significant reduction in TSWV infection as well as enhancement of growth of tomato plants both in the glasshouse and field conditions. Increased activity of polyphenoloxidase (PPO), β-1,3-glucanase and chitinase in BCA-treated tomato plants was observed. The presence of a new protein was detected only in BCA-treated plants. Enzyme-linked immunosorbent assay (ELISA) indicated that the viral antigen concentration was reduced in parallel to disease intensity (Kandan et al. 2005).

Pseudomonas chlororaphis strain 06 suppressed the effects of *Cucumber mosaic virus* (CMV) infection in tobacco. The role of global regulator GacS in the strain 06 in stimulating growth promotion and ISR in tobacco was investigated. Root colonization of cv. Samsum with wild-type 06 and the *gacS*-complemented mutant elicited resistance and reduced the intensity of symptoms and titer of CMV. In tobacco cv. GX3, disease intensity was not altered, following root colonization by the wild-type, *gacS* mutant or complemented mutant. But the viral titer was reduced in plants colonized by wild type and the *gacS* mutant strains. The plant growth was not adversely affected by CMV infection in plants treated with wild-type and *gacS* mutant. The bacterial BCA induced systemic resistance in tobacco against CMV without a negative impact on growth (Ryu et al. 2003). *Banana bunchy top virus* (BBTV) causes the destructive bunchy top disease in banana. Strains of *P. fluorescens* CHA0 and Pf1 were able to reduce the incidence of the disease and promote the growth of the plants as well. The bioformulation of these bacterial strains either alone or in combination with chitin were evaluated for their potential to induce systemic resistance against BBTV in greenhouse and field conditions. The bioformulation containing CHA0 amended with chitin stimulated the defense-related enzymes in treated plants challenged with BBTV under greenhouse conditions.

The viral antigen concentration was reduced in BCA-treated plants in proportion to the disease severity. The enhanced growth of BCA-treated plants was reflected in higher bunch yield (Kavino et al. 2008). The effects of treatment of banana with bioformulations containing *Pseudomonas fluorescens* (Pf1) and *Bacillus* sp. (EPB22) on the development of *Banana bunchy top virus* (BBTV) infection were assessed. The disease incidence was found to be reduced by 80 and 52 % respectively in the greenhouse and field evaluations. The virus titer was significantly reduced in plants treated with the bacterial mixture as indicated by enzyme-linked immunosorbent assay (ELISA). Biochemical investigations showed that PR-protein synthesis and activities of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase were activated. In addition, accumulation of phenolic compounds was observed in bacterized banana plants, indicating that the reduction in banana bunchy top disease was likely to be due to in induction of systemic resistance by the BCA mixture. Enhanced plant growth and increase in yield were additional benefits due to the application of bacterial strains (Harish et al. 2009).

5.1.1.6 Factors Influencing Mechanisms of Biocontrol

Application of biocontrol agents has been favored as an alternative crop disease management strategy in place of chemical application. However, the lack of consistency in the performance of bacterial biocontrol agents under field conditions has limited their practical use in commercial agriculture. The inconsistency has been attributed to a greater extent to variability in physical and chemical properties within niches occupied by the biocontrol agents that influence both colonization and expression of various mechanisms of biocontrol activities against microbial pathogens leading to ineffective suppression of disease development.

The method employed to apply bacterial BCAs for treating the seeds may affect the distribution and pattern of colonization of bacteria and subsequently the efficacy of the BCA against seedborne pathogens. It is essential to have the information on parts of the seeds to be colonized for achieving effective biocontrol. Molecular techniques like GFP tagging have been used to monitor the pattern of colonization of bacteria. The *gfp* gene encoding the green fluorescent protein (GFP) was employed to tag *Pseudomonas chlororaphis* MA342 effective against *Drechslera teres,* causing seedborne netblotch disease of barley. The *gfp*-tagged strain MA342 G2 had the same biocontrol potential as the wild-type strain, when it was applied at high cell concentrations to seeds, but was less effective at lower concentrations. The number of culturable cells was significantly less than the total number of bacterial cells on seeds which were dried for 20 h after inoculation. Confocal microscopy and epifluorescence stereomicroscopy were used to determine the pattern of MA342G2 colonization and cell aggregation of barley seeds. Immediately after bacterization of seeds, the BCA cells were seen mainly under the seed glume and no specific pattern could be observed. After the seeds were sown, aggregation of bacteria towards and near the embryo, but not in the embryo was recognizable. Aggregates of bacteria were commonly present in the groove of each seed formed by the base of the

coleoptile and the scutellum. The results suggested that the strain MA342 might colocalize with the pathogen, facilitating the antifungal compound (2,3-deepoxy-2,3-didehydrorhizoxin) produced by the BCA to reach the target pathogen propagules (Tombolini et al. 1999).

Many abiotic factors existing in soil such as pH, temperature, moisture, texture and inorganic and organic constituents can significantly influence the activities of biocontrol agents. Temperature is one of the important factors, influencing both colonization of roots by rhizobacterium, as well as the expression of their biocontrol mechanisms. In the case of *Fusarium oxysporum* f.sp. *ciceris* race 5, incubation temperature and inoculum density of the pathogen strongly interacted in modulating the expression of Fusarium wilt in chickpea by four rhizobacteria viz., *Pseudomonas fluorescens* RGAF19, *P. fluorescens* RG26, *Bacillus megaterium* RGAF51 and *Paenibacillus marcesans* RGAF101. When the conditions became favorable for wilt disease development, the effectiveness of biocontrol by the rhizobacteria decreased (Landa et al. 2001). In a later study, seed and soil treatment with *P. fluorescens* isolates RGAF 19 and RG26, significantly increased chickpea shoot dry weight at 20 °C and root dry weight at 25 and 30 °C. They colonized the chickpea rhizosphere and internal stem tissues at 20, 25 and 30 °C and there was a positive linear trend between bacterial population size in the rhizosphere and temperature increase. The maximum inhibition of mycelial growth and conidial germination of *F. oxysporum* f.sp. *ciceris* race 5 in vitro occurred at a temperature range optimal for bacterial growth and production of toxic metabolites like pyoverdin (Landa et al. 2004).

The knowledge of interactions between crop cultivars, pseudomonads and soil types may be useful to optimize cultivar-soil combinations for the promotion of growth through beneficial BCAs. Three Swiss winter wheat (*Triticum aestivum*) cultivars Arina, Zinal and cimetta were characterized for their ability to accumulate naturally-occurring plant-beneficial pseudomonads in the rhizosphere. The ability to select for specific genotypes of 2,4-DAPG producers in two different soils was used as the basis to determine the cultivar performance cultivar, specific differences were strongly influenced by the soil-type. The *phlD* diversity among the *Pseudomonas* spp. substantially varied between the two soils, as indicated by the denaturing gradient gel electrophoresis (DGGE) analysis of fragments of the DAPG biosynthetic gene *ph1D* amplified from natural *Pseudomonas* rhizosphere populations. Further, there was a cultivar-specific accumulation of certain *phlD* genotypes in only one soil. Among the three cultivars tested, Arina was protected most effectively against *Pythium ultimum* infection by treatment with *P. fluorescens* CHA0. However, in terms of growth promotion, this cultivar derived the least benefit from the interaction with the BCA, in the absence of pathogen infection. The results suggested that it might be possible to improve the plant-beneficial effects of root colonizing pseudomonads by breeding wheat genotypes with greater potential to sustain interactions with the PGPR (Meyer et al. 2010).

Bacterial species/strains have been reported to promote fungal spore germination and/or root colonization by arbuscular mycorrhizal (AM) fungi. The bacteria promoting the establishment of mycorrhizal symbiosis (ecto-and endo-mycorrhizas) by increasing root-fungus contacts and colonization are designated mycorrhizae

helper bacteria (MHB). *Pseudomonas fluorescens* C7R12 and BEG12, the cell organization of C7R12 was found earlier to promote colonization of *Medicago truncatula* roots by *Glomus mosseae* BEG12. To understand the underlying mechanism of interaction between C7R12 and BEG12, the cell organization of C7R12 was characterized on adventitious roots mycorrhized or not with BEG12 and extraradical hyphae by employing the immunofluorescence technique and confocal laser scanning microscope. Bacterial cells more frequently remained single on mycorrhizal than on non-mycorrhizal roots and in microcolonies and strings on mycorrhizal roots. In addition, the root area covered by bacterial cells, as revealed by image analysis, appeared to be significantly lower on mycorrhizal than on non-mycorrhizal roots. Cells of C7R12 were abundant on extraradical hyphae and organized both as single cells and microcolonies. The results suggested that *P. fluorescens* C7R12 cells became less active and were found to be less abundant on mycorrhizal than on nonmycorrhizal roots (Pivato et al. 2008).

The effectiveness of the biocontrol by introduced bacteria relies primarily on their ability to maintain stable populations and to remain metabolically active in the rhizosphere. It is known that the sizes of introduced pseudomonad populations may decline appreciably within a few weeks and consequently the beneficial effects of the introduced bacteria tend to be variable. Among the biological factors affecting the biocontrol activity, bacteriophages which are ubiquitous in the soil environment are potentially important. They are considered to be important as an ecologically important factor with negative impact on bacterial populations. The practice designated phage therapy involves the use of bacterial viruses that can only infect specific bacteria by lysing the susceptible cells of the bacterial species/strains/pathogens. A lytic bacteriophage ØGP100 that could specifically infect *Pseudomonas fluorescens* CHA0 and some closely related *Pseudomonas* strains were isolated from soil. The influence of phage ØGP 100 on the biocontrol potential strain CHA0 and its rifampicin resistant derivative CHA0-Rif was assessed. The phage ØG100 has double-stranded DNA as the genome with an icosahedral head and stubby tail. In the presence of ØGP100, the population size of the strain CHA0-Rif in soil and on cucumber roots was reduced by more than 100-folds. As a result, the biocontrol potential to protect cucumber against *Pythium ultimum* was entirely abolished. However, the phage did not affect either root colonization and/or the disease suppression by a ØG100-resistant variant of the strain CHA0-Rif. The results suggested that the emergence of a phage resistant subpopulation strain CHA0-Rif might occur too slowly to allow for the build up bacterial population that might effectively suppress *P. ultimum* that infects the plant roots at very early stages of growth (Keel et al. 2002). Introduction of *lux* genes into bacterial strains and measurement of the resulting bioluminescence can provide a sensitive marker to track introduced rhizobacteria in nonsterile soil environments and to estimate their physiological activity in situ (White et al. 1996). Root colonization and in situ bioluminescence are considered to be indicators of biocontrol activity in the rhizosphere of plants. Bioluminescence was strongly correlated with dehydrogenase of activity of *Pseudomonas fluorescens* in in vitro cultures. In situ bioluminescence indicated the physiological activity of *Pseudomonas fluorescens* B5. Colonization of the roots at ≥4 cm below the seed

decreased at very low soil water matric potential (-330×10^3 Pa). Total population size of the strain B5 per seedling was significantly increased at -140×10^3 Pa. However, matric potential had no significant effect on the bacterial population density per g of root fresh weight and did not affect the distribution of the population down the root. Total population size per seedling and downward colonization by the strain B5 were significantly reduced at high temperatures (25–35 °C). Antagonistic activity of *P. fluorescens* B5 against *Pythium ultimum* causing beet root damping-off disease decreased with increasing soil temperature and decreasing matric potential (Schmidt et al. 2004).

5.1.2 Bacillus *spp.*

The use of Gram-positive *Bacillus* spp. as biocontrol agents has been relatively less frequent compared with the Gram-negative *Pseudomonas* spp., despite the fact that the strains of *Bacillus* spp. are widely distributed, have high thermal tolerance and grow very rapidly in liquid cultures. The genus *Bacillus* includes several bacterial species such as *B. subtilis, B. cereus, B. amyloliquefaciens, B. pumilus, B. mycoides, B. pastueri* and *B. sphaericus* which suppress development of diseases affecting a wide range of crop plants. The antagonistic activity of *Bacillus* spp. against crop pathogens may be due to antibiosis, competition for nutrients and space, hyperparasitism and induced systemic resistance to diseases. Antibiosis is the mechanism of biocontrol activity of most *Bacillus* spp. and in many cases the precise mechanism of biocontrol activity of bacterial biocontrol agents remains unclear. *Bacillus* spp. produce dormant spores that are resistant to desiccation, heat, UV irradiation and organic solvents. The ability to produce the endospores and various antibiotics makes the *Bacillus* spp. to be attractive candidates suitable for formulation and commercialization. Many commercial products containing *Bacillus* spp. as the active ingredient have been launched.

5.1.2.1 Metabolites-Mediated Antagonism

Antibiotics

Most of the spore-forming members of *Bacillus* spp. produce antibiotics that are low molecular weight peptides produced via the non-ribosomal biosynthetic pathway which involves specific enzymes known as peptide synthetases. The peptides exhibit a broad-spectrum of biological activities including antifungal, antibacterial, antiviral and antitumoral activities (Emmert et al. 2004). The metabolites with antimicrobial properties may be produced via ribosomal biosynthetic pathway also (Zuber et al. 1993). *B. subtilis* was reported to synthesize about 60 different types of antibiotics, many of which have antifungal properties of the compounds belonging to the iturin family (Phae and Shoda 1991). Detection of antibiotic

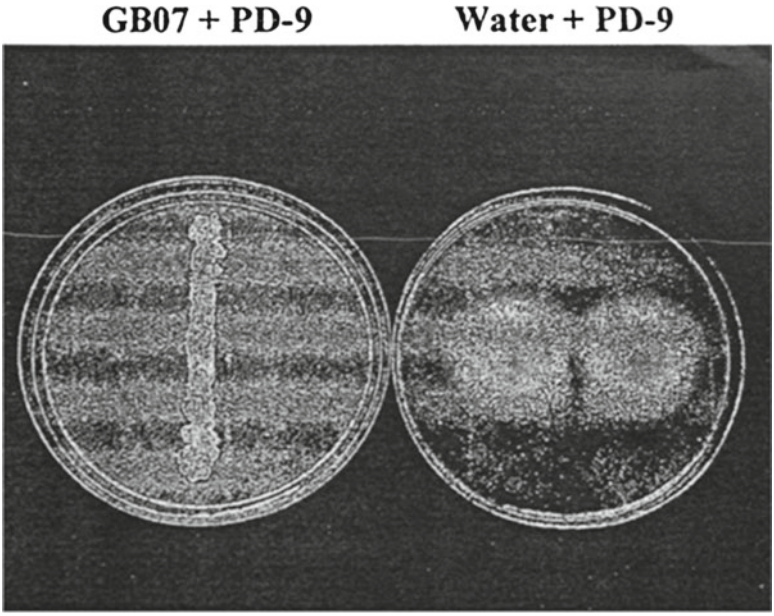

Fig. 5.5 In vitro assessment of antibiotic activity of *Bacillus subtilis* GB07 against *Penicillium digitatum*, causative agent of green mold disease of orange (Courtesy of Zhang and Dou 2002 and with kind permission of Florida State Horticulture Society, Florida, USA)

production by the bacterial isolates is important in determining their biocontrol potential in suppressing the plant pathogens and the diseases caused by them. Various biochemical and molecular methods have been employed to detect and characterize the secondary metabolites produced by *Bacillus* spp.

Cultural Methods

The antibiotic activity of *Bacillus subtilis* strain GB07 against the post- harvest pathogen *Penicillium digitatum,* causing green mold of oranges was assessed in vitro. The strain GB07 was streaked on potato dextrose agar (PDA) medium at the center of the plate. After 2 days of incubation, aliquots of 10 µl of the pathogen conidial suspension (10^6 spores/ml) were dispensed on either side of the bacterial streak at a distance of 2 cm. Suitable control plates streaked with sterile water and inoculated with pathogen were maintained. The antibiotics secreted by the strain GB07 strongly suppressed the growth of *P. digitatum,* as there was no visual mycelial growth (Fig. 5.5) (Zhang and Dou 2002). The antagonistic activity of *Bacillus* spp., filtered culture filtrates and selected secondary metabolites was assessed in vitro against *Xanthomonas campestris* pv. *campestris* (*Xcc*) causal agent of cabbage black rot disease. All three species *B. amyloliquefaciens, B. subtilis* and *B. pumilus* were found to be efficient metabolic producers, when growing in half-strength trypticase soy broth (TSB) or in cabbage broth (CB). Generally TSB stimulated

Table 5.6 Production of secondary metabolites by three species of *Bacillus* in tryptic soy broth and cabbage broth at 30 °C (Wulff et al. 2002)

	Bacillus spp.		
Secondary metabolite matches	*B. amyloliquefaciens*	*B. subtilis*	*B. pumilus*
Surfactin	12/18[a]	17/19	13/14
Iturin	11/18	2/19	–
Bacillomycin	9/18	1/19	–
Azalomycin F	15/18	–	–
Amphomycin	1/18	1/19	10/14
Acivicin	3/18	5/19	2/14
Anthrobactin	8/18	14/19	8/14
Rhodotorola acid	7/18	5/19	–
Valinomycin	3/18	1/19	12/14
Stenothricin	1/18	2/19	5/14
Colistin	–	–	3/14
Enterochelin	1/18	–	1/14
Nocardamin	2/18	–	–

[a]Number of isolates producing the designated antibiotic/total number of isolates tested
–Absence of detectable amounts of the designated antibiotic

greater production of metabolites compared with control. *B. amyloliquefaciens* isolates produced surfactin, iturin, bacillomycin and/or azalomycin F, while *B. subtilis* isolates were mostly able to synthesize surfactin and arthrobactin. The culture filtrates of *B. pumilus* isolates contained surfactin, amphomycin, arthrobactin and valinomycin. All three species produced surfactin in the cultures (Table 5.6) (Wulff et al. 2002). *B. subtilis* strains GB-017 and GB-0356 isolated from the soil were able to inhibit the mycelial growth of the fungal pathogens *Botrytis cinerea*, *Fusarium* sp., *Pythium* sp. and *Rhizoctonia solani.* The disc diffusion method was employed to assess the antifungal activity of the bacterial strains. The antagonistic activity was maintained up to pH 9.0 and remained stable at 80 °C for 1 h. The antifungal compounds were purified using ion-exchange and adsorption columns and the exhibited characteristics corresponded to polyenes and lactones (Kim et al. 2003).

The potential of antibiotic production by *Bacillus subtilis* effective against soybean seed pathogenic fungi was assessed. *B. subtilis* strain PRBS-1 (from soybean rhizosphere) and AP-3 (reference strain from rice rhizosphere) were compared for their efficacy in their antifungal and growth promotion activities. Both strains inhibited the mycelial growth of the fungal pathogens *Rhizoctonia solani*, *Colletotrichum truncatum, Sclerotinia sclerotiorum, Macrophomina phaseolina* and *Phomopsis* sp. pathogenic to soybean. The antibiotic effect of compounds purified from the culture filtrates appeared to be similar to the compounds of iturin group. The growth promotion effect of the strain PRBS-1 was at least partially related to the production of indole-acetic acid. The results indicated that the biocontrol potential and growth-promoting activity of *B. subtilis* strain PRBS-1 may be useful for effective management of soybean seed pathogenic fungi (Araujo et al. 2005). *B. amyloliquefaciens* strain Bg-C31 produced an antimicrobial substance which was identified as a protein with resistance to heat and protease K activity. The antagonistic gene was located

in the chromosome by plasmid curing. A 29-kDa protein encoded by the *LC1* gene was expressed. The antimicrobial activity of the fusion protein to *Ralstonia solanacearum* was detected. The antibacterial protein showed the potential for suppressing the Capsicum bacterial wilt disease and the pathogen causing the disease (Hu et al. 2010).

The ability of *Bacillus subtilis* to inhibit the mycelial growth of *Fusarium verticillioides* causing Fusarium head blight disease of wheat and to inhibit accumulation of fumonisin B1 was assessed in vitro. All strains (10) of *B. subtilis* significantly inhibited the pathogen growth, the strain CE1 exhibiting greatest antifungal activity. The strains CE1 and 86 only were able to reduce the mycotoxin production by 50 and 29 % respectively. The strain CE1 was the only strain capable of inhibiting the growth of and toxin production by *F. verticillioides*. The antagonistic activity of the strain CE1 was assessed in the greenhouse to find a link with the results obtained in vitro. There was significant antibiotic activity (60 %) and reduction of *F. verticillioides* population (40–50 % in CFU) in the in vitro assays. A similar biocontrol efficacy was observed also in the greenhouse experiments which showed inhibition of colonization reaching high values (98.55–99.86 %) (Cavaglieri et al. 2005). *Bacillus subtilis* strain EIR-j, an endophyte isolated from wheat roots showed high antifungal activity against *Gaeumannomyces graminis* var. *tritici* (*Ggt*), causative agent of wheat take-all disease. Soil drenches with bacterial cell densities of 10^6, 10^9 and 10^{12} CFU/ml reduced the infection by *Ggt* in wheat seedlings by 62.6, 68.6 and 70.7 % respectively at 4 weeks after sowing. Treatment with the strain EIR-j increased the growth and yield parameters, compared to untreated wheat plants and *Ggt*-inoculated plants. EIR-j treatment alleviated the deleterious effects of take-all on grain parameters to an extent similar to that of fungicide Triadimefon. Observations with scanning electron microscope (SEM) showed that in the presence of the bacterial strain, hyphae of *Ggt* showed leakage, appeared ruptured, swollen and shriveled. Examination under transmission electron microscope (TEM) revealed that cells of EIR-j were present in the root tissues of wheat seedlings and effectively retarded infection and colonization of *Ggt* in root tissue. Disintegration of pathogen hyphal cytoplasm occurred, following the suppressive activity of the BCA strain. In addition, formation of wall appositions of papillae, representing morphological defense reactions triggered by the bacterial strain could also be observed in the treated root tissues of wheat inoculated with the pathogen (Liu et al. 2009a, b).

The biocontrol potential of two bacterial BCAs *Bacillus subtilis* (MAB) and *Paenibacillus polymyxa* (MAP) obtained as two commercial products was assessed against *Phytophthora capsici*, causal organism of pepper (chilli) Phytophthora blight disease. *B. subtilis* inhibited the mycelial growth of *P. capsici.* On the other hand, *P. polymyxa* inhibited formation of zoosporangium by *P. capsici.* Release of zoospores from zoosporangium was significantly inhibited by both BCAs. The results indicated that the secondary metabolites of these BCAs target the pathogen at different stages of its life cycle (Kim et al. 2010). *Bacillus subtilis* CMB32 isolated from soil, was able to inhibit the mycelial growth of *Colletotrichum gloeosporioides*, causative agent of anthracnose diseases. In addition, this strain was antagonistic also to other fungal pathogens such as *Fusarium solani, Botrytis*

cinerea, F. oxysporum, Rhizoctonia solani and *Phytophthora capsici*. The strain CMB2 produced antifungal biosurfactant lipopeptides iturin A, fengycin and surfactin A which were detected by MALD1-TOF mass spectrophotometry. Iturins and fengycins exhibited powerful antifungal activity and growth inhibition of several fungal pathogens. Surfactins were not toxic by themselves, but sustain some synergistic effect on the antifungal activity of iturin A (Pyoung II et al. 2010).

Strains of *Bacillus subtilis* produce cyclic lipopeptides which constitute the iturin family which are powerful antifungal agents. *B. subtilis* strain KS1 was isolated from grape berry skin and it was identified as a new strain based on the morphological, biochemical and genetic analyses. The strain KS1 suppressed the mycelial growth of the gray mold pathogen *Botrytis cinerea* and grape ripe rot pathogen *Colletotrichum gloeosporioides.* Under field conditions KS1 reduced the grape downy mildew caused by *Plasmopara viticola* on berry skins and leaves. The KS1 genome had *ituD* and *Ipa-14* genes which were shown to have a role in the biosynthesis of iturin A. The mutants lacking both the genes lost the antagonistic activity of the parent strain KS1 against *B. cinerea* and *C. gloeosporioides* and lacked the ability to produce iturin A. The results suggested that the antagonistic activity of KS1 against grapevine fungal pathogens might depend on the ability to produce iturin A. The strain KS1 was found to be tolerant to various pesticides. Hence, the application of chemicals, either prior to or after treatment with KS1, can be included as a component of integrated disease management system for grapevine diseases (Furuya et al. 2011). The isolate SB10 of *Bacillus* spp. reduced the incidence of Phytophthora blight disease of pepper caused by *P. capsici* by 72.2 %. The antifungal compounds produced by SB10 were identified as lipopeptide complex, namely surfactins, iturins and fengycins, by employing Matrix-assisted Laser Desorption Ionization-Time of Flight (MASDI-TF) mass spectrometry technique (Oh et al. 2011).

Bacillus amyloliquefaciens strain RC-2 was able to effectively suppress the development of mulberry anthracnose disease caused by *Colletotrichum dematium*. The culture filtrate (CF) of the strain RC-2 suppressed the appearance of anthracnose lesions significantly, only when applied prior to inoculation of cabbage with *C. dematium,* but not after pathogen inoculation. This suggested that the antifungal compounds secreted by the strain RC-2 had only a preventive effect on the disease. Addition of peptone significantly enhanced the production of antifungal compounds by the bacterial strain. Observations under the scanning electron microscope (SEM) showed that the conidial germination was entirely inhibited on mulberry leaves pretreated with undiluted CF of RC-2. The CF could also inhibit the growth of other plant pathogens such as *Pyricularia oryzae. Xanthomonas campestris* pv. *campestris* and *Agrobacterium tumefaciens.* Different kinds of antifungal compounds were isolated by high performance liquid chromatography (HPLC) analysis. Iturin A2, a cyclic peptide was one among the antifungal compounds identified by nuclear magnetic resonance and fast atom bombardment mass analysis (Yoshida et al. 2001). *Bacillus amyloliquefaciens* strain MET0908 isolated from soil was found to be effective suppressing the development of *Colletotrichum lagenarium,* causing anthracnose disease infecting leaves, stems and fruits of watermelon. The strain

MET0908 produced antifungal compounds in cocultures, but not when grown in pure cultures. The supernatant of the coculture with *C. lagenarium* inhibited the mycelial growth of the pathogen. An antifungal protein was purified by 30 % ammonium sulfate saturation and it was concentrated using Centricon 10, DEAE- Sepharose™ Fast Flow column and Sephacryl S-100 gel filtration chromatography. The purified protein with a MW 40-kDa inhibited the growth of several other plant pathogens like *Fusarium graminearum, Colletotrichum gloeosporioides, Pythium ultimum, Phytophthora capsici, Didymella bryoniae* and *Monosporascus cannonballus*. The BCA strain secreted an extracellular β-1,3-glucanase that acted on fungal cell walls. Confocal image analysis microscopy showed that the antifungal protein was embedded in the septa of the hyphal wall of *C. lagenarium.* Scanning electron microscopy revealed abnormal swelling, degradation and burst by the excretion of lytic enzymes of the BCA. The mode of action of the antifungal protein was by the disruption of the pathogen cell wall. The antifungal proteins secreted by *B. amyloliquefaciens* strain MET 0908 showed no significant homology with any known proteins (Kim and Chung 2004).

Bacillus amyloliquefaciens strain DGA 14 isolated from the surface of banana fruits was able to inhibit the mycelial growth of the fungal pathogens *Thielaviopsis paradoxa, Colletotrichum musae*, *Fusarium verticillioides,* and *Lasiodiplodia theobromae* which collectively cause the postharvest crown rot disease of banana. The strain DGA 14 produced a diffusible metabolite that inhibited all fungal pathogens tested in culture. The bacterial strain moved and attached to fungal pathogens significantly suppressing mycelial growth and inhibited conidial germination in liquid medium. In addition, the bacterial strain parasitized the fungal pathogens effectively. DGA14 survived and colonized banana fruits after 2 days. The incidence of crown rot disease of banana was significantly reduced by the application of the strain DGA 14 which was more effective than the fungicide used to treat the banana fruits in the packing house (Alvindia and Natsuaki 2009). The endophytic bacteria *Bacillus amyloliquefaciens* isolated from poplar (*Poplus* spp.) trees showed antimicrobial activity against fungal and bacterial pathogens with variable efficacy. The strain PEBA 20 effectively reduced infection by *Botryosphaeria dothidea,* causing canker disease in poplar. Aberrant hyphae were observed after the treatment of *B. dothidea* with bacterial suspension or fermentation filtrates. The hyphal aberrations increased with treatment duration, exhibiting bead-like appearance and cluster-like structures at the hyphal tips. Cut shoots dipped in bacterial suspension (10 CFU/ml) for 30 min had reduced incidence of canker (60 %), compared to controls (100 %). Delay in canker development and reduction in lesion size were also recorded in treated shoots. As *B. amyloliquefaciens* is known to produce a range of antifungal dipeptides or cyclic peptides, the antagonistic activity of the strain PEBA 20 could be due to its ability to secrete antifungal metabolites capable of inducing abnormalities of pathogen hyphae, resulting in reduction in canker incidence in cut shoots, following treatment with bacterial suspension/culture filtrates (Yin et al. 2011).

Bacillus licheniformis strain P40 inhibited the development of *Erwinia carotovora* causing soft rot disease in stored potatoes. The strain P40 produced a novel

bacteriocin-like substance (BLS) which exhibited bactericidal effect on *E. carotovora* cells at 30 µg/ml. The effect of treatment of pathogen cells with BLS was assessed using transmission electron microscope (TEM). The BLS-treated cells showed wrinkled bacterial surfaces and shrinkage of whole bacterial cell, indicating plasmolysis. Treatment of potato tubers with BLS, at a concentration of 240 µg/ml or higher, substantially reduced the symptoms of soft rot and the symptom development was completely arrested at a concentration of 3.7 mg/ml of BLS. The results showed the effectiveness of the strain P40 and its metabolite for control of potato soft rot disease (Cladera-Olivera et al. 2006). The endophytic *Bacillus vallismortis* ZZ185 isolated from healthy stems of Broadleaf Holly (*Ilex latifolia*) showed strong antagonistic activity against *Fusarium graminearum, Alternaria alternata, Rhizoctonia solani, Cryphonectria parasitica* and *Phytophthora capsici* in vitro, when exposed to the culture filtrate and n-butanol extract of this strain. The antifungal activity of the culture filtrate (CF) was significantly correlated with cell growth of the BCA strain. The secondary metabolite of the BCA was relatively heat stable with more than 50 % of the antifungal activity of the CF being retained after exposure to a temperature of 121 °C for 30 min. The antifungal activity of the CF against the mycelial growth of *A. alternata* and *F. graminearum* remained almost unaltered, even after exposure to a range of pH from 1 to 8. The antifungal compounds were purified from the n-butanol extract of the CF and they were identified as a mixture of bacillomycin D (*n*-C14) and bacillomycin D (iso-15). This bacterial strain ZZ185 of *B. vallismortis* appears to have potential for the management of fungal diseases of several crops (Zhao et al. 2010).

Biochemical and Physiological Methods

The antibiotic producers of *Bacillus* isolates were identified and grouped by applying fatty acid methyl ester (FAME) analysis. The fatty acid methyl esters were extracted from each isolate using standard procedures for gas chromatographic (GC) FAME analysis. The isolates *B. amyloliquefaciens* were identified by FAME analysis. These isolates produced surfactin, iturin, bacillomycin and/or azalomycin. Their ability to inhibit *Xanthomonas campestris* pv. *campestris* (*Xcc)* causal agent of cabbage black rot disease, varied considerably from weak to a very strong effect. However, no general relationship was observed between in vitro inhibition and biocontrol effect. Isolates identified by FAME analysis as *B. pumilus* were molecularly very heterogeneous. The isolates varied substantially in their ability to inhibit *Xcc* in vitro and their biocontrol activity in vivo was not related to the in vitro growth inhibition effect on the pathogen (Wulff et al. 2002). *B. luciferensis* strain KJ2 C12 isolated from pepper roots, was subjected to FAME analysis. The FAME profiles of this strain were composed mainly of anteiso 15:0 and iso IS:0 which were similar to that of *B. marinus* (similarity = 0.535 %). The identity of this BCA was, however, established based on the similarity of 16S rDNA sequences. *B. luciferensis* effectively protected pepper plants against Phytophthora blight disease caused by *Phytophthora capsici* (Kim et al. 2009).

Molecular Techniques

Cultural methods are time-consuming and labor-intensive in providing results. On the other hand, molecular methods may be useful for rapid identification of antibiotic-producing strains of bacteria. The strains of *Bacillus* obtained from soils naturally suppressive to plant diseases and other substrates could be screened using polymerase chain reaction (PCR)-based methods. *Bacillus subtilis* and four strains of *Bacillus* sp. were screened by PCR amplification, using degenerate primers corresponding to peptide synthetase genes. Four isolates of *Bacillus* sp. gave positive amplification signal and three isolates exhibited inhibitory activity against the mycelial growth of *Sclerotinia sclerotiorum*, indicating that the PCR- based techniques could be employed for rapid selection of antibiotic-producing strains of *Bacillus* spp. (Giacomodonato et al. 2001). Suppressive substrative hybridization (SSH) was employed to identify genetic markers associated with biological control activity of by *B. subtilis* against microbial plant pathogens. Sixteen subtracted fragments with high degree of similarity to sequences present in several strains of *B. subtilis* with known biocontrol potential were selected. Oligonucleotide primers specific to nine of these genes were designed. The targeted genes included five genes involved in antibiotic synthesis (*bmyB, fenD, ituC, srfAA* and *srfAB*) and four additional genes. All nine markers were amplified from three commercialized *B. subtilis* strains with the exception of *ituC*. The strains positive for amplifiable markers generally were more effective in inhibiting the growth of *Rhizoctonia solani* and *Pythium ultimum.* The presence of amplifiable markers in the isolates of *Bacillus* spp. may be used as a basis of selecting the isolates rapidly (Joshi and McSpadden Gardener 2006).

Screening of potential strains of *Bacillus* spp. with antibiotic and biocontrol activities for the presence of specific antibiotic-encoding sequences by employing molecular methods forms a rapid approach in comparison with traditional method of selection. The presence of antibiotic biosynthetic genes for the antibiotics surfactin, iturin A, bacillomycin D, fengycin, mycosubtilin, and zwittermicin A by specific PCR using 21 *Bacillus* spp. which showed >50 % mycelial inhibition or leaf/stem infection reduction. MALDI-TOF-MS method is an easy and less time-consuming method for confirming the production of antibiotics, compared with other available procedures. The PCR products amplified from all strains using the primer pair SUR3F/3R showed a 441-bp band corresponding to the surfactin antibiotic biosynthetic gene. The antibiotic biosynthetic gene for iturin A was detected in all strains except the strain 3039, using the primer pair ITUDIF/IR. The presence of bacillomycin D biosynthetic genes could be detected only in strains BS6, 3059 and 4079. Two primer pairs had to be employed for the detection of zwittermycin A in the strains BS6 and BS8. The strains possessing the multiple genes BS6, BS8, H-08-02, S-07-01, 3057 and 4079 along with other positive strains were subjected to MALDI-TOF-MS analysis. The antibiotics produced by four strains are presented in Table 5.7. Production of antibiotics by some strains could not be recognized by MALDI-TOF-MS, even in the presence of relevant genes were detected by PCR assay. All isolates of *B. subtilis, B. mycoides, B. amyloliquefaciens, B. licheniformis* and *B. cereus/thuringiensis* were positive for

Table 5.7 Production of antibiotics by *Bacillus* spp. as determined by MALDI-TOF-MS analysis (Athukorala et al. 2009)

Bacterial BCA/strain	Antibiotics produced
Bacillus amyloliquefaciens BS6	Surfactins, bacillomycin D, fengycins
B. mycoides 4079	Surfactins, bacillomycin D, iturin A,, fengycin
B. subtilis 3057	Surfactins, bacillomycin D, fengycin
B. subtilis H-08-02	Surfactins, iturin A, fengycin

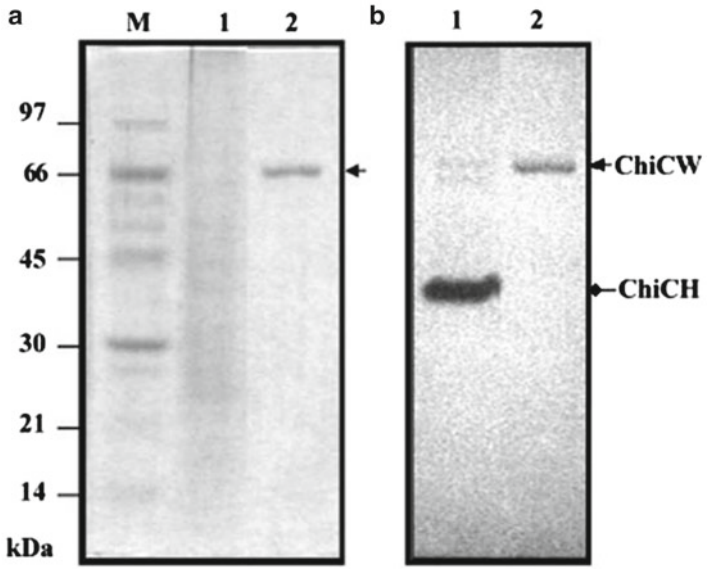

Fig. 5.6 Separation of two chitinase ChiCW and ChiCH secreted by *Bacillus cereus* 28–9 using SDS-PAGE technique. (**a**): gel stained with Coomassie Brilliant Blue; (**b**): gel stained with Calcoflour White M2R; *M* low molecular weight protein standard (Courtesy of Huang et al. 2005 and with kind permission of Journal of Biochemistry and Molecular Biology)

surfactin and iturin A. The presence of biosynthetic genes for and production of fengycin and surfactin by *B. mycoides* has been reported for the first time (Athukorala et al. 2009).

Hydrolytic Enzymes

Various species and strains of *Bacillus* have been demonstrated to produce antibiotics and/or enzymes in the cultures either naturally or stimulated in the presence of required substrates. *B. cereus* has been shown to be a reliable BCA of soybean Phytophthora damping-off and root rot disease. The cotton root rot disease caused by *Rhizoctonia solani* was suppressed by an endophytic strain of *B. cereus* (Pleban et al. 1997; Emmert et al. 2004). *B. cereus* 28–9 exhibited high biocontrol activity against Botrytis leaf blight of lily. This bacterial strain produced at least two chitinases ChiCW and ChiCH which could be extracted from the culture filtrates (Fig. 5.6).

Table 5.8 Effect of purified preparation of ChiCW on the conidial germination of *Botrytis elliptica* (Huang et al. 2005)

Activity of ChiCW (μU)[a]	Inhibition rate of conidial germination (%)
28.0[a]	84 ± 1
14.0	78 ± 3
7.0	29 ± 8
3.5	20 ± 2
0.0	0

[a]A mixture of 4 μl of purified ChiCW at different concentrations and 4 μl of conidial suspension of *B. elliptica* (10^5 conidia/ml) incubated for 12 h prior to microscopic determination of conidial germination

The purified enzymes inhibited the conidial germination of *Botrytis elliptica.* The conidia of *B. elliptica* incubated with ChiCW became enlarged and formation of germ tubes was retarded (Huang et al. 2005). In a later study, a chitinase-secreting *B. cereus* strain CH2 was isolated from the rhizosphere of eggplant. Based on the activity and purification, using SDS-PAGE technique, the enzyme was identified as a 15.0-kDa chitinase. On glass slides, germination of the fungal spores was effectively suppressed by the bacterial suspension, supernatant from the suspension, and 0.005 % solution of chitinase extracted from the strain CH2. The chitinase required pH 7.1 and 40 °C for its optimal activity. In greenhouse assays, the severity of Verticillium wilt disease of eggplant was reduced by 69.69, 54.04 and 53.13 % respectively by cell suspension, supernatant and diluted enzyme preparation (0.01 %). This strain CH2 appeared to have good commercial potential for the control of eggplant Verticillium wilt (Table 5.8) (Li et al. 2008a, b).

Various kinds of ion-exchange and adsorption chromatography were employed for estimating antifungal activity of *B. subtilis* against *Botrytis cinerea* and other fungal pathogens. Thin layer chromatography (TLC) procedure was used to separate the antifungal compounds which had properties corresponding to polyene and lactone (Kim et al. 2003). *B. amyloliquefaciens* effective against *Colletotrichum lagenarium* causing anthracnose disease of watermelon produced an antifungal compound in the cultures grown in potato dextrose broth. The cell-free filtrates were concentrated by centrifugation. Solid ammonium sulfate was used to precipitate proteinaceous substances. DEAE-Sepharose™ Fast Flow Column was used for further purification by employing sodium dodecylsulpahte (SDS)- polyacrylamide gel electrophoresis (PAGE) method. A single band with a molecular weight of about 40-kDa showed antifungal activity against *C. lagenarium* and other plant pathogens such as *F. graminearum, Pythium ultimum* and *Phytophthora capsici*. The purified antifungal protein was identified as β-1-3-glucanase. *B. amyloliquefaciens* strains MET 0908 exhibited strong activity against *C. lagenarium* (Kim and Chung 2004). *Bacillus subtilis* NSRS 89–24 has been shown to be effective against two important fungal pathogens, causing rice blast disease (*Magnaporthe grisea*) and rice sheath blight disease (*Rhizoctonia solani*). The

bacterial strain inhibited the growth of *M. grisea* and *R. solani* in vitro. NSRS 89–24 produced a heat stable antibiotic and labile enzyme β-1,3-glucanase. The glucanase activity in the culture medium of NSRS 89–24 strain was inducible in the presence of chitin (0.3 %), reaching the maximum activity at 5 days after incubation. The targets for the antifungal compounds were in the cell wall of fungi at each hyphal apex which is composed of chitin, β-glucans and other oligosaccharide compounds. The β-glucanase activity of *B. subtilis* NSRS 89–24 might have an important role in the degradation of pathogen cell wall. The extracellular β-1,3-glucanase produced by NSRS 89–24 was purified and the molecular mass was estimated. Both purified enzyme and the antibiotic could separately inhibit the growth of *M. grisea* and *R. solani*. The results indicated that glucanase and antibiotic could act in concert, exhibiting, strong synergistic effect on both rice pathogens. Production of these bioactive compounds in vivo has to be demonstrated (Leelasuphakul et al. 2006).

Bacillus cereus strain UW85 produced two antibiotics, zwittermicin A and kanosamine in its culture supernatant. Zwittermicin A is a water soluble, acid-stable, linear aminopolyol molecule with broad-spectrum activity against numerous fungal and bacterial pathogens (He et al. 1994). Later *Bacillus* spp. have been shown to produce lytic enzymes such as chitinases, proteases or glucanases that may have a role in their biocontrol activities against plant pathogens. *B. cereus* strain AU 004 isolated from soil samples was evaluated for its ability to produce hydrolytic enzymes. This bacterial strain secreted complex of hydrolytic enzymes such as chitinase, chitosanase and protease, when grown in a medium containing chitosan flakes of marine waste. The culture supernatant significantly inhibited the growth of *Fusarium oxysporum*, *F. solani* and *P. ultimum*. The protease from the culture supernatant was purified by sequential chromatography and characterized as a neutral protease with MW 28.8-kDa, and optimal pH and temperature for protease activity at 7 and 50 °C respectively. The purified protease inhibited both spore formation and the hyphal development of *P. ultimum* in vitro, indicating the role of the enzyme in the biocontrol activity of *B. cereus* AV004 against the fungal pathogen. Isolation of a protease from *Bacillus* spp. appears to have been achieved for the first time in this investigation (Chang et al. 2009).

Bacillus subtilis strain CHU26 isolated from potato field exhibited strong extracellular chitinase activity on the colloidal chitin-containing agar plate. The strain CHU 26 strongly inhibited the mycelial growth of *Rhizoctonia solani*. The gene encoding chitinase (*chi18*) was cloned from the constructed *B. subtilis* CHU26 genomic DNA library. The *chi18* gene consisted of an ORF of 1,791 nucleotides and encoded 595 amino acids with a MW of 64-kDa. The amino acid sequence of the chitinase gene showed 62 and 81 % similarity to those from *B. circulans* WL-12 and *B. licheniformis* respectively. *Escherichia coli* was transformed with *chi18* gene and the transformant exhibited chitinase activity on colloidal chitin agar plate as the CHU26 strain. The transformant reduced infection of radish seedlings by *R. solani* by more than 90 %, indicating the role of chitinase as an important mechanism in the biocontrol activity of *B. subtilis* strain CHU26 (Yang et al. 2009). *Bacillus pumilus*

strain SG2 effective against the fungal pathogens *Fusarium graminearum* and *Bipolaris sorokiniana*, produced two different chitinases in the presence of colloidal chitin. The chitinases inhibited the mycelial growth of *F. graminearum* and caused abortion of hyphal elongation of *B. sorokiniana.* In contrast, expression of chitinases by the strain SG2 was repressed, when glucose was used as the carbon source and the antifungal activity of *B. pumilus* strain SG2 was consequently abolished. The hyphal inhibition of *F. graminearum* and *B. sorokiniana* by the bacterial strain remained stable for a minimum period of 14 days. These results confirmed that expression of the *B. pumilus* SG2 chitinases was under the control of two types of regulation, special regulation of chitin and global regulation by glucose. The results revealed that activity of chitinases was the principal mechanism of the biocontrol activity of *B. pumilus* SG2 against the wheat pathogens (Shali et al. 2010).

5.1.2.2 Prevention of Colonization of Plant Tissues by Pathogens

The principal mechanism of biocontrol activity of *Bacillus* spp. against plant pathogens may be through production of antibiotics and/or hydrolytic enzymes that act adversely on the pathogens directly. Rhizosphere colonization by *Bacillus* spp. was reported to suppress root disease in cotton (Mahaffe and Backman 1993). Root infection by *Fusarium verticillioides* results in systemic invasion of maize plants, leading to the accumulation of the mycotoxin, fumonisins in maize grains and products. The influence of *B. subtilis* CE1 on native *F. verticillioides* colonization at different inoculum concentrations and maize root levels was investigated. Bacterization of maize seeds with CE1 strain at 10^6–10^8 cells/ml inoculum concentration inhibited *F. verticillioides* counts at the rhizosphere levels, whereas all bacterial treatments reduced the fungal CFUs at the endorhizosphere level. The highest bacterial concentration provided the maximum level of inhibition percentages of *F. verticillioides* infection of maize roots. The results indicated the ability of *B. subtilis* CE1 to reduce rhizosphere and endorhizosphere colonization by the pathogen *F. verticillioides,* when the strain CE1 was applied as seed inoculants (Cavaglieri et al. 2005). The ability of four *Bacillus* spp. isolated from vegetable crops such as sugar beet, tomato and potato was assessed for colonizing cocoa (*Theobroma cacao*) seedlings and reducing the severity of black pod rot disease caused by *Phytophthora capsici*. The bacterial strains exhibited differential ability to colonize cocoa leaves. The strains BT8 and BP24 of *B. cereus* were able to establish long-term colonization, whereas the strains BacJ (of *B. mycoides)* and 203–7 (of *B. pumilus*) could colonize the cocoa leaves for short periods, their populations declining at different rates. Total foliar colonization in plants treated with BT8 and BP24 was relatively at high levels. In the leaf disk bioassay, only the strain BT8 was able to suppress the expansion of lesions induced by *P. capsici*, although the strain BP24 could colonize the disk tissues. Foliar colonization of leaves by these strains was primarily epiphytic with limited endophytic invasion. Suppression of *Phytophthora* by BT8 occurred in non-colonized plants. The results suggested that

systemic resistance might be induced in other plant parts by colonization of leaves of cocoa (Melnick et al. 2008).

Bacillus subtilis strain EXWB1 isolated from healthy melon (*Cucumis melo* L.) fruits has been demonstrated to be a unique mechanism of biocontrol activity against *Alternaria alternata,* infecting melon fruits during transport and storage. The droplet of the strain EXWB1, when placed on melon skin, spread as a thin film on the hydrophobic fruit surface, indicating that the bacterial strain established effective attachment to the fruit surface. Further the rate of EXWB1 colony extension on melon skin was 125 μm/h, suggesting that the bacterial strain could rapidly occupy the same niche as the fungal pathogen. Production of biosurfactant was found to be useful for attachment of the bacterial cell to the fruit surface. The hyphae of *A. alternata* could not grow on melon skin or wounds inoculated with EXWB1 cell suspension and no rotting of the fruit flesh in fruits treated with bacterial cell suspension could be seen. Ethylene is known to accelerate fruit senescence. Treatment of melon fruits with EXWB1 cell suspension delayed ethylene production by 2 days and the amount of ethylene produced was also reduced. In addition, the respiration of melon fruits treated with EXWB1 cells suspension was suppressed, when challenged with the pathogen inoculation. EXWB1 inoculation was beneficial in maintaining the fruit firmness which was drastically affected by *A. alternata* in untreated control fruit. As this bacterial strain existed on healthy fruit surfaces, the potential of EXWB1 could be advantageously exploited for protecting melon fruits under storage conditions (Wang et al. 2010).

5.1.2.3 Induction of Resistance to Crop Diseases

Elicitation of induced systemic resistance (ISR) to diseases infecting various crops by strains of *Bacillus* has been demonstrated in greenhouse or field trials on cucumber, muskmelon, watermelon, tomato, bell pepper, sugar beet, and tobacco. Different species of *Bacillus, B. subtilis*, *B. amyloliquefaciens*, *B. cereus, B. pumilus, B. mycoides, B. pastuerii* and *B. sphaericus* have been evaluated for their ability to elicit ISR resulting in significant reduction in the incidence and/or severity of diseases on a wide range of host plant species. Elicitation of ISR by *Bacillus* spp. was associated with ultrastructural changes in plants and cytochemical alterations during pathogen attack. Activation of some of the same pathways as in *Pseudomonas* spp. and some additional pathways has been demonstrated in different pathosystems. ISR elicited by several strains of *Bacillus* spp. was found to be independent of salicylic acid (SA), but dependent on jasmonic acid (JA), ethylene (ET) and the regulatory gene *NPR1*. Nevertheless, other strains induced ISR dependent on SA and independent of JA and *NPR1*. Further, ISR induced by *Pseudomonas* spp. does not result in accumulation of *PR1* gene in plants. On the other hand, ISR induced by *Bacillus* spp. in some cases led to accumulation of *PR1* gene in treated plants. Promotion of plant growth by treatment with *Bacillus* spp., in addition to disease suppression, has been found to be an added advantage (Kloepper et al. 2004).

In the roots of pea bacterized with *Bacillus pumilus* strain SE34, challenged with *Fusarium oxysporum* f.sp. *pisi* (*Fop*), colonization of *Fop* was restricted to endodermis and paratracheal parenchyma cells and radiated towards the vascular stele. The restriction of pathogen in SE34-treated plants was due to strengthening of the epidermal and cortical cell walls. In addition, cell wall appositions with large amounts of callose and infiltrated with phenolic compounds were also present in the root tissues of bacterized pea plants (Benhamou et al. 1996). This bacterial strain induced systemic resistance in tomato plants against *F. oxysporum* f.sp. *lycopersici* (*Fol*) resulting in reduction in severity of typical symptoms and number of brown lesions formed on lateral roots of treated tomato plants. By using gold-complexed β-1,3-glucanase assay, higher amounts of β-1,3-glucans in the root tissue were detected, when plants were treated with strain SE34 and chtiosan, compared with individual treatments and untreated control plants (Benhamou et al. 1998). In tobacco-blue mold (*Peronospora tabacina*) infected tobacco plants treated with *Bacillus pumilus* strain SE34, significantly increase in the levels of salicylic acid (SA) at 1 day post-inoculation with *P. tabacina* was observed (Zhang et al. 2000).

A detached leaf and microtiter plate bioassays were developed to assess elicitation of ISR by bacterial BCAs strains. Application of *Bacillus pasteuri* C-9 and *B. pumilus* SE34 and T4 as soil drenches to three tobacco cultivars reduced significantly the mean percentage of leaf area with lesions induced by *Peronospora tabacina*, in addition to reduction in the sporulation of the pathogen. Disease severity was substantially reduced by strains SE34 and T4, but not by strain C-9, as determined by detached leaf and microplate assays (Zhang et al. 2002). In a later study, the efficacy of modes of BCA application, seed treatment and/or soil drench in inducing ISR was determined. The strains SE34 and C-9 significantly enhanced the growth of tobacco as seed treatment. ISR was elicited only by C-9, but not by SE34 or T4. As seed treatment the T-4 had no effect either on growth enhancement or ISR induction in plants. However, when the bacterial strains were applied as seed treatment followed by soil drenches, all strains promoted the plant growth. When the interval between the last application of bacteria and challenge inoculation with *P. tabacina* was 6 weeks, all bacterial strains could induce ISR. The results suggested that plant growth promotion and elicitation of ISR by tested bacterial strains may be linked with each other (Zhang et al. 2004).

Induction of systemic resistance can be demonstrated through challenge assays in which distal, untreated leaves are challenged with a pathogen, following a short priming period with an inducing on a primary, spatially separated leaf or root system (Conrath et al. 2002). *Bacillus mycoides* strain Bac J reduced the severity of Cercospora leaf spot disease of sugar beet caused by *Cercospora beticola.* The leaf was sprayed with BacJ strain was bagged. The plant was challenge-inoculated by spraying the conidial suspension of *C. beticola* (10^8 CFU/ml) after bacterial treatment of bagged leaf. The disease severity was significantly reduced on both highly susceptible and moderately resistant sugar beet varieties (Bargabus et al. 2002). The ability of *Bacillus mycoides* isolate BmJ and *B. mojavensis* isolate 203–7 in suppressing the development of cucumber anthracnose disease caused by *Glomerella*

cingulata var. *orbiculare* by inducing systemic acquired resistance (SAR) in treated plants. The isolates BmJ and 203–7 delayed disease incidence and reduced total and live conidial production per mm^2 of lesion area significantly relative to the untreated control plants. Cucumber apoplastic proteins were assayed at 6 days after induction. The isolates BmJ and 203–7 enhanced β-1,3-glucanase activities by 135 and 72 % respectively. The isolate 203–7 also increased peroxidase activity by 79 % over control treatment. In the field trials, the isolate BmJ applied 1 week before inoculation reduced significantly the AUDPC (=0.05) in cucumber, compared to water control in 1 year (2004) and on cantaloupe for both years (2004 and 2005). The disease severity was reduced by 41 % in cucumber and >20 % in cantaloupe levels equal to that obtained by using fungicides azoxystrobin and chlorothalonil (Neher et al. 2009).

In a later study, treatment of sugar beet leaves with live BacJ strain or avirulent strain of *Erwinia carotovora* pv. *betavasculorum* (*Ecb*) resulted in significant control of leaf spot disease. In addition, this treatment resulted in a two-fold increase in chitinase- and β-glucanase-specific activity, indicative of systemic resistance induction. Hypersensitive cell death was induced by avirulent *Ecb*, but not by BacJ strain. An oxidative burst elicited by spray application of BacJ strain under both light and green light conditions was not dependent on the stomata for entry into the sugar beet tissues. The BacJ strain elicited an oxidative burst in sugar beet similar in timing, but not in intensity to that elicited during incompatible interaction. The oxidative response was observed, only following live BacJ strain cell treatment, a requirement for effective disease control as well (Bargabus et al. 2003). *Bacillus cereus* selected from 500 rhizobacteria isolated from soil, rhizosphere and rhizoplane of healthy tomato plants, was found to be effective against foliar fungal and bacterial pathogens infecting tomato. After the removal of bacterial cells by centrifugation, the supernatant was dialyzed repeatedly. The dialysates, when applied to the roots, protected the tomato plants against diseases caused by fungi and bacteria. The results indicated that the macromolecules secreted by *B. cereus* could act as elicitors of systemic resistance in treated tomato plants against diseases (Romeiro et al. 2005).

The efficacy of *Bacillus amyloliquefaciens* IN937a, *B. subtilis* GB03 and a mixture of these two strains in reducing the severity of cucumber angular leaf spot disease due to *Pseudomonas syringae* pv. *lachrymans* was assessed. Seed treatment with bacterial BCAs reduced severity of disease and it increased plant growth also significantly by inducing systemic resistance (Raupach and Kloepper 2000). *Bacillus pumilus* strain SE34 incorporated into the potting medium provided systemic protection to tomato against the late blight disease caused by *Phytophthora infestans*. The chemical inducer of disease resistance DL-β-amino-*n*-butyric acid (BABA) was applied as a foliar spray to one half of the nonbacterized plants as a positive control at 4 weeks after seeding. The plants were challenge-inoculated with the pathogen after 1 week. The disease severity was expressed as the percentage of leaves covered with late blight lesions. Treatment with strain SE34, significantly reduced the severity of late blight disease. In addition, the plant height and overall plant weight were increased in plants treated with the strain SE34 compared with untreated control plants (Yan et al. 2002, 2003). *Bacillus amyloliquefaciens* strain

BS6 and *Pseudomonas chlororaphis* strain PA-23 were evaluated for their potential in reducing the incidence of stem rot disease of canola under greenhouse and field conditions caused by *Sclerotinia sclerotiorum*. The effectiveness of these BCA strains in controlling the canola stem rot disease was in equivalence to the fungicide Rovral Flo ® (iprodione). The suppression of stem rot disease development by the BCA strains was achieved by reducing canola flower petal infection by *S. sclerotiorum* through both direct antimicrobial action and/or induction of plant defense enzymes (Fernando et al. 2007).

The mechanism of biocontrol activity of *Bacillus pumilus* 7 km isolated from wheat rhizosphere against *Gaeumannomyces graminis* var. *tritici* causing take-all disease, was studied. The soil was drenched with bacterial cell suspension and changes in the defense-related enzymes and total phenolics contents were determined. Disease severity was significantly reduced in the bacterized roots of wheat plants and plant growth was promoted markedly due to treatment. The activities of soluble peroxidase (SPOX), ionically cell wash bound peroxidase (CWPOX), β-1,3-glucanase, β-1,4-glucanase, were increased in plants treated with *B. pumilus.* In addition, the phenolic contents of treated plants also showed higher levels, compared with that of untreated control plants. The enzyme activities reached the peak at 4–8 days after application of BCA to the wheat roots. The disease suppressive activity of *B. pumilus* might be linked to its ability to stimulate the plant's defense systems operating in the root system locally or systemically (Sari et al. 2007). The ability of *Bacillus subtilis* strains S2BC-1 and GIBC-Jamog was assessed as biocontrol agents against tomato wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici*. Seed bacterization and soil application of the mixture of these two strains reduced the wilt disease incidence significantly as determined by localized and split-root-experiments. Enhanced activities of chitinase and β-1,3-glucanase was observed in root samples from plants treated with the bacterial strains. High intensity peroxidase isoforms could be detected in gels for samples from localized and ISR experiments. The results indicated the possibility of mechanisms of both direct antibiosis and induction of ISR operating in concert in tomato plants protected by the BCA strains (Shanmugam and Kanoujia 2011).

The secondary metabolites secreted by *Bacillus* spp. were evaluated for their ability to induce ISR in treated plants/organs. *Bacillus* spp. produce different lipopeptide antibiotics. Treatment of potato tuber cells with purified fengycins resulted in the accumulation of phenolic compounds involved in or derived from phenylpropanoid metabolism (Ongena et al. 2005b). The purified surfactin of *B. subtilis* S499 was able to induce systemic resistance against *Botrytis cinerea* in bean and tomato plants. In tomato cells, activation of key enzymes of lipoxygenase pathway appeared to be activated in resistant plants, following induction by surfactin-overproducing isolates (Ongena et al. 2007). Similar reaction was observed in canola plants receiving double application of *Pseudomonas chlororaphis* strain PA23 on canola petals. The activity of chitinase and β-1,3-glucanse was enhanced resulting in the suppression of *Sclerotinia sclerotiorum* (Fernando et al. 2007). Blackleg disease caused by *Leptosphaeria maculans* (anamorph: *Phoma lingam*) is another economically important disease of canola. *Bacillus cereus* strain DFE4 and

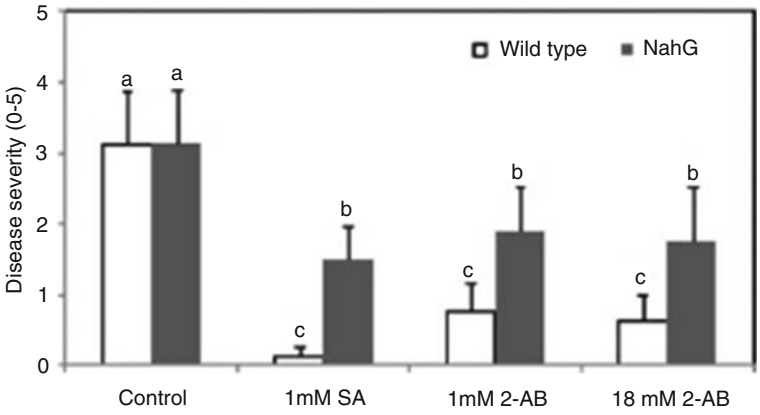

Fig. 5.7 Induction of systemic resistance in wild type and NahG transgenic tobacco in wild type and NahG transgenic tobacco by 2-AB from *Bacillus* sp. BS107 against *Pectobacterium carotovorum* subsp. *carotovorum* SSC1 2-AB was applied at 1.0 and 18.0 mM on 3-week old Xanthi nc tobacco and NahG transgenic tobacco seedlings. *Different letters on bars* indicate significant differences between treatments according to Fisher's protected LSD test at P = 0.05 (Courtesy of Yang et al. 2010 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

B. amyloliquefaciens strain DFE16 produced the lipopeptide antibiotics iturin A, bacillomycin D and surfactin. The results indicated that the direct antifungal activity of the antibiotics was the most dominant of black leg disease control by the bacterial BCAs. However, low but significant disease suppression from induced resistance also occurred (Ramarathnam et al. 2011).

Bacillus sp. strain BS107 was able to elicit ISR against soft rot disease of tobacco caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). A determinant of ISR secreted by the strain BS107 was isolated from the cell-free culture supernatant and identified by mass spectrometry and NMR analyses as 2-aminobenzoic acid (2-AB) as a principal ISA determinant. 2-AB displayed effective ISR activity against soft rot disease development on the tobacco leaves. Treatment of tobacco roots with the 2-AB exhibited protective effects against *Pcc*. No inhibition of the pathogen occurred at the concentration of 2-AB that induced resistance. Reverse transcription (RT)-PCR assays of tobacco leaves of plants treated with 2-AB on the roots, showed up-regulation of the induced resistance marker genes such as *PR1a PR1c, PR2* and *PR4*. 2-AB is biosynthesized from chorismic acid which is a precursor for salicylic acid (SA). It is well known that SA has a vital role in mediating plant defenses. Salicylic acid treatment at 1.0 mM significantly reduced tobacco soft rot disease severity as 0.75. In contrast, it lost the capacity, when NahG plants were treated. Application of 2-AB at 1.0 and 18.0 mM into NahG plants resulted in significant increase in the symptom severity to 2.5- and 2.8-fold respectively than that was observed in wild type plants (Fig. 5.7). The results indicated that 2-AB protected systemically NahG tobacco against *Pcc* and 2-AB-elicited ISR required SA signaling. Colonization of BS107 on plant root system could play a role in

promoting plant defenses, as it secreted bacterial determinants including 2-AB for protecting plants against challenge by diverse pathogens (Yang et al. 2010).

Demonstration of induction of systemic resistance by root-colonizing bacteria to diseases caused by soilborne pathogens may be difficult, since the requirement of spatial separation of ISR-eliciting bacterium and the pathogen may not be satisfied in some pathosystems. Treatment of cucumber with *B. pumilus* strain INR7 was reported to induce ISR against cucurbit wilt disease caused by *Erwinia tracheiphila*. The number of wilted leaves per plant was reduced in INR7-treated cucumber plants challenged with inoculation of the pathogen by natural insect vectors, striped cucumber beetle (*Acalymma vittatum*) and spotted cucumber beetle (*Diabrotica undecimpunctata*). Elicitation of systemic resistance by the INR strain was observed under field conditions, where the bacterial strain was applied as seed treatment and soil drench (Zehnder et al. 1997). In a later study, bacterial strain INR7 was applied as seed treatment and drenches, while preparing transplants. The mean percentage of wilted vines was significantly reduced in plants treated with strain INR7 and insecticide, compared with untreated control plants. The bacterial treatment was not significantly different from the level of protection obtained from insecticide treatment (Zehnder et al. 2001).

Antibiotic production by bacterial BCAs has been shown earlier to be primarily responsible for suppression of several crop pathogens. But several strains of *Bacillus* spp. and *Pseudomonas* spp. have been shown to be capable of inducing systemic resistance to crop pathogens. *B. subtilis* strain BSCBE4 and *P. chlororaphis* PA23 were able to induce systemic resistance against *Pythium aphanidermatum* in hot pepper (chilli) and also to promote the growth of treated plants. Defense-related enzymes phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO) were stimulated, in addition to accumulation phelolic compounds. Incidence of damping-off was significantly reduced due to treatment with the bacterial BCA strains (Nakkeeran et al. 2006). *B. subtilis* M4 was able to stimulate a systemic response in cucumber and tomato leading to protection against *Colletotrichum lagenarium*, causing anthracnose disease of cucumber and *Pythium aphanidermatum*, inducing damping-off disease of tomato seedlings. The pathogen and M4 strain were spatially separated from each other, thus excluding the possibility of antibiotic secreted by the bacteria having a role in disease control. Treatment of plant roots with M4 strain was found to induce systemic molecular modifications. The RNA expression profiles in control and treated plant leaves were compared using the cDNA-AFLP technique. Several AFLP fragments corresponded to genes, not expressed in control plants treated with M4 strain. The differential accumulation of mRNA indicated the plant reaction following perception of M4 strain. Evidence for the specific modulation of gene expression by *B. subtilis* strain in tomato and cucumber triggering plant defense machinery was established in this investigation (Ongena et al. 2005a).

The biocontrol potential of strains (20) of *Bacillus* spp. for suppression of development of *Penicillium digitatum*, causing the citrus green mold disease was assessed. Volatile compounds of 9 of 20 strains tested, inhibited the mycelial growth of the pathogen by more than 80 %. The ethanol extract from *B. subtilis* 155 cell-free

supernatant containing secondary metabolites (SMs) exhibited maximum inhibitory effect on mycelial growth and spore germination of *P. digitatum* with EC_{50} values of 77.26 and 82.10 µg/l respectively. The protein separated from the SMs by ammonium sulfate precipitation had an EC_{50} of 288 µg/l. The antifungal protein activity was associated with lowest band obtained after polyacrylamide gel electrophoresis (PAGE) procedure. Inoculation with 20 µl of a 10^8 CFU/ml of *B. subtilis* endospore suspension at 24 h, prior to fungal spore inoculation, decreased infection of *P. digitatum* by 86.7 % and production of disease symptoms was delayed by 6 days and decay symptoms by 9 days. Addition of SMs solution (10 µg/ml) simultaneously with the pathogen, decreased disease incidence by 72.5 % and symptom appearance was delayed by 5 days after inoculation. No sign of decay could be seen up to 9 days. The results indicated that antifungal compounds produced by *B. subtilis* 155 might have an important role in suppressing the growth of the green mold pathogen infecting citrus fruits (Leelasuphakul et al. 2008).

The ability of *Bacillus cereus* strain CIL to induce systemic resistance in *Lilium formosanum,* when applied as soil drench against leaf blight disease caused by *Botrytis elliptica*, was assessed. Protection provided by the bacterial strain was for about 10 days against the leaf blight disease. *B. cereus* strain CIL was assayed for ISR-eliciting activity and root colonization trait. A 40 % reduction in disease severity could be achieved, when the bacterial strain suspension was applied to the rhizosphere. The bacterial strain could effectively colonize the roots of lily, maintaining a population of over 10^4 CFU/cm at 10 days after application. The expression of *LfGRP1* and *LsGRP1* genes encoding glycine-rich protein associated with *L. formosanum* and oriental lily cv. Stargazer respectively was analyzed. *LsGRP1* was found to be an SA-dependent pathogen-inducible gene. *LfGRP1* expression showed that it could be induced by the pathogen. Both *LfGRP1* and *LsGRP1* transcripts decreased after the application of the BCA strain. The expression of *LfGRP1* and *LsGRP1* were extensively suppressed, when *B. elliptica* was inoculated on CIL-treated lily plants. The negative regulation was considered to be controlled by a signal transduction pathway induced by *B. cereus* strain CIL that differed from the pathogen-induced SA-directed pathway (Liu et al. 2008a, b).

The physiological responses of lily cv. Star Gazer with induced systemic resistance, triggered by *Bacillus cereus* CIL against *Botrytis elliptica* were studied by using histological and biochemical analyses. Leaves inoculated with *B. elliptica* displayed cell death, H_2O_2 accumulation and lignin deposition. In plants treated with *B. cereus,* cell death and H_2O_2 accumulation and lignin deposition in leaves caused by *B. elliptica* infection were suppressed, indicating that suppression of oxidative burst might be associated with *B. cereus* C12-induced systemic resistance. In reactive oxygen species inhibitors assays, *B. elliptica*-induced lesion numbers and H_2O_2 accumulation in lily leaves were significantly reduced in leaves pretreated with catalase or diphenylene iodonium. In addition, the expression of *LsGRP1* and *LsPsbR* in leaves, elicited with *B. cereus* CIL and inoculated with *B. elliptica,* was decreased. Similar expression pattern was also observed in leaves pretreated with catalase or diphenylene iodonium and inoculated with *B. elliptica.* The results suggested that *B. cereus*

CIL-induced systemic resistance might be related to suppression or alleviation of oxidative stress and cell death of lily caused by *B. elliptica* (Huang et al. 2012).

Bacillus vallismortis strain EXTN-1 isolated from red pepper was able to effectively suppress tomato bacterial wilt disease caused by *Ralstonia solanacearum*. *Bacillus subtilis* strain 816–6, *B. pumilus* strain 228–7, *Bacillus* sp. strain 113–3 and *Paenibacillus polymyxa* strain H32-5 were also evaluated along with strain EXTN-1 for their efficacy against *R. solanacearum*. The tomato seedlings roots were bacterized with the bacterial strains and the tomato plants were grown in perlite-hydroponic system. Upon challenge inoculation with the pathogen, all bacterial strains suppressed the development of bacterial wilt disease. *B. vallismortis* strain EXTN-1 was the most effective, in reducing the infection to 65 % as against 95 % infection in untreated control plants. The movement of the pathogen from the site of inoculation was hampered in plants treated with the strain EXTN-1. As the strain EXTN-1 did not have any direct antagonistic activity against *R. solanacearum*, the suppression of disease development was probably achieved by a mechanism other than antibiosis. The strain EXTN-1 produced an efficient elicitor for inducing systemic resistance in many crops (Park et al. 2007). The bacterial BCA mixture of strains containing *Bacillus atrophnius* S2BC-2 and *Burkholderia cepacia* TEPF-Sungal protected the gladiolus plants against *Fusarium oxysporum* f.sp. *gladioli* causing vascular wilt and corm rot disease to the maximum extent. The protection by these strains was attributed to induction of systemic resistance as deduced from the stimulation of PR-proteins and activities of PO, PPO and PAL in addition to phytoalexins and/or chalcone synthase (Shanmugam et al. 2011). *Bacillus pumilus* strain INR-7 was able to reduce the incidence of pearl millet downy mildew disease caused by *Sclerospora graminicola* by inducing systemic resistance in treated seedlings. Resistance induced by the strain INR-7 was associated with the expression of hypersensitive response (HR), enhanced lignifications, callose deposition and hydrogen peroxide as well as increase in expression of defense-related enzymes such as β,1,3-glucanase, chitinase, phenylalnine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO). Microscopical examination of inoculated pearl millet tissues revealed accumulation of lignin, callose and hydrogen peroxide earlier and to higher levels in resistant and induced resistant seedlings. Tissue print analysis revealed localization of defense- related enzymes in the vascular bundles and difference in the expression pattern of β-1,3-glucanase, chitinase, PAL, PO and PPO in genetically resistant, INR-7-treated and susceptible pearl millet seedlings (Raj et al. 2012).

The ability of *Bacillus cereus* AR156 to provide systemic protection to tomato against bacterial speck disease caused by *Pseudomonas syringae* pv. *tomato* DC 3000 and root knot caused by *Meloidogyne incognita* and to promote plant growth was assessed. The strain AR156 population reached 10^5–10^6 CFU/g in the rhizosphere of tomato plants for more than 2 months. The bacterial BCA increased the average biomass of tomato plants by 47.7 %. AR156 elicited induced systemic resistance against DC3000, resulting in reduction in disease severity by 1.6 fold and inhibition of pathogen proliferation by approximately 15-fold. DC 3000 strain

triggered accumulation of defence-related genes (*PR1* and *PIN2*) in tomato leaves and primed the leaves for accelerated defense-related gene expression upon challenge with DC 3000. The results suggested that simultaneous activation of salicylic acid (SA) and jasmonic acid dependent signaling pathways by AR156 against DC 3000. The bacterial strain AR 156 was able to form robust colonies in the roots of tomato and exerted same beneficial effects, including suppression of development of bacterial speak disease via ISR and plant growth promotion (Niu et al. 2012).

Plant growth-promoting rhizobacteria (PGPR) have been demonstrated to enhance the levels of resistance to several fungal and bacterial diseases infecting plants. The ability of PGPR to induce resistance to virus diseases of plants has been assessed in some pathosystems. PGPR-mediated induced resistance against *Tobacco necrosis virus* (TNV) and *Cucumber mosaic virus* (CMV) was demonstrated earlier (Maurhofer et al. 1994; Raupach et al. 1996). The potential of *Bacillus* spp. strains to induce systemic resistance to *Cucumber mosaic virus* (CMV) was tested by treating the seeds of cucumber and tomato with single strains of bacteria under greenhouse conditions. Treatment with bacterial strains significantly reduced the area under disease progress curve, when the cotyledons of plants were inoculated mechanically with CMV. There was delayed symptom development in some of the treated plants (Zehnder et al. 2000). The ability of *Bacillus* spp. to reduce the incidence and severity of disease caused by *Tomato mottle virus* (ToMV) transmitted by whiteflies was assessed, by employing combination of bacterial strains under field conditions, when high populations of whitefly vectors were available. The presence and titre of ToMV was determined by quantitative estimation of ToMV DNA. Treatment with bacterial strains resulted, in lower percentage of infected plants and larger yields, compared with non-bacterized control plants (Murphy et al. 2000). The efficacy of combination of *Bacillus subtilis* GB03 plus any one of the strain of *Bacillus pumilus* (SE34, INR7, T4), *B. amyloliquefaciens* (IN 937a) and *B. subtilis* (IN937b) formulated with the carrier chtiosan was assessed in enhancing resistance to CMV infection and growth of tomato. Tomato plants treated with the biopreparations, each of which contained two bacterial strains, appearing similar to the untreated controls which were 10 days older, were challenged with CMV. The CMV disease severity ratings were significantly lower and treated plants had better growth than the control plants of same age at 14 and 28 days post-inoculation (dpi). Accumulation of CMV in young noninocualted leaves was much less in all biopreparation-treated plants except the plants treated with the combination of T4 strain, as revealed by the virus concentration assessment by enzyme-linked immunosorbent assay (ELISA). The protection provided by biopreparation appeared to have resulted from the enhanced growth of tomato plants, thereby allowing them to respond to inoculation with CMV in a manner similar to that of more mature plants exhibiting adult plant resistance, a phenomenon frequently observed in different virus-host combinations (Murphy et al. 2003).

Several isolates of bacteria obtained from plant samples were screened for their antiviral activity against *Tobacco mosaic virus* (TMV). The isolate ZH14 identified as *Bacillus cereus* secreted antiviral compounds which inhibited virus infection by 94.2 %. The isolate ZH14 had the ability to degrade the ribonucleic acid. The antiviral product of ZH14 has an extracellular protein with high molecular mass with

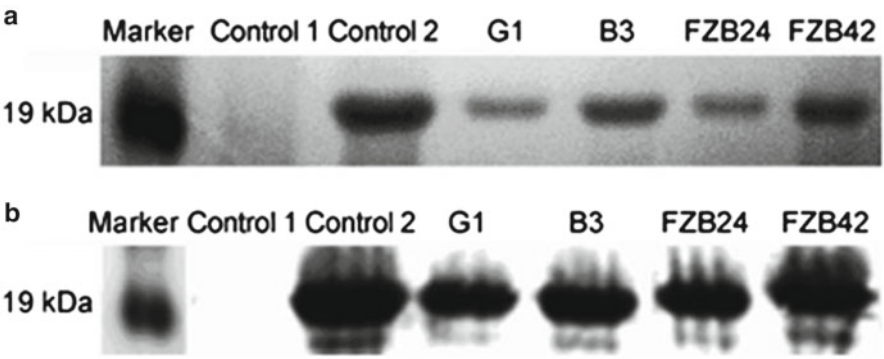

Fig. 5.8 Detection of *Tobacco mosaic virus (TMV)* coat protein (*CP*) in tobacco treated with different PGPR strains at 28 days post-inoculation (*dpi*). (**a**): SDS-PAGE analysis; (**b**): Western blot analysis. *Control 1*: tobacco without TMV inoculation; *Control 2*: tobacco inoculated with TMV and treated with water G1, B3, FZB24 and FZB42 represent tobacco treated with bacterial strains and inoculated with TMV (Courtesy of Wang et al. 2009 and with kind permission of Korean Society for Microbiology and Biotechnology, South Korea)

temperature optima between 15 and 60 °C and pH optimum of 6–10. The results indicated that the BCA strain had the potential to remain antagonistic to TMV at a wide range of temperature and pH conditions (Zhou et al. 2008). *Bacillus subtilis* strain G1 and B3 and *B. amyloliquefaciens* strains FZB24 and FZB42 were evaluated for their potential for inducing systemic resistance to *Tobacco mosaic virus* (TMV) and improving plant growth in tobacco plants under greenhouse conditions through application of the bacterial strains as seed treatment. The bacterial strains significantly reduced disease severity induced by TMV, as indicated by visible symptoms and reduced virus accumulation as revealed by ELISA tests. The amount of coat protein (CP) (20-kDA) was significantly lower in plants treated with *Bacillus* strains (Fig. 5.8). The plants treated with G1 and F2B24 strains contained lower levels of TMV-CP than that was present in B3- and FZB42-treated plants. The *NPR1* gene is a regulator in the expression of various sets of defense genes involved in the SAR and ISR resistance pathways. The gene *Coi1* positively regulates the JA-dependent ISR pathway. An RT-PCR analysis of the signaling regulatory genes *Coi1* and *NPR1* and defense genes *PR-1a,* and *PR-1b* in treated tobacco leaves revealed an association with enhancement of systemic resistance of tobacco to TMV. The results of this investigation provided evidence indicating the disease protection to tobacco by *Bacillus* strain G1 was based on plant-mediated ISR. In addition, *Bacillus* strains stimulated the growth of tobacco plants substantially that could be recognized both in the roots and leaves of tobacco. Thus treatment of tobacco with *Bacillus* strains was able to enhance the systemic resistance to TMV and to promote the growth of tobacco as well (Wang et al. 2009).

The efficacy of *Bacillus pumilus* strain EN16 and *B. subtilis* strain SW1 in inducing systemic resistance in tobacco against *Tobacco mosaic virus* (TMV) was assessed under greenhouse conditions. Treatment with strain EN16 and SW1 significantly reduced the intensity of mosaic symptoms and disease severity, resulting in 52 and 71 % protection at 14 days after inoculation respectively. Virus titer in tobacco was

determined by employing the enzyme-linked immunosorbent assay (ELISA). The bacterial BCA-treated plants showed decrease in TMV concentration. A period of 5 and 7 days interval between inducer treatment and challenge inoculation with TMV was required for induction of resistance respectively by EN16 and SW1. The activities of phenylalanine ammonia lyase (PAL), peroxidase, polyphenol oxidase and pathogenesis-related (PR) proteins were assessed in tobacco plants treated with BCA strains and inoculated with TMV. The amounts of defense-related enzymes and PR-proteins significantly increased in *Bacillus*-treated plants challenged with pathogen, when compared with control (Lian et al. 2011). *Bacillus subtilis* strains are able to induce resistance to virus diseases of crops and to promote growth of the treated plants. The gene encoding the $HPaG_{xooc}$ protein from the rice pathogenic bacterium *Xanthomonas oryzae* pv. *oryzicola* which elicited hypersensitive cell death in non-host plants, thereby inducing resistance to diseases and pests. The strain OKB105 of *B. subtilis* was transformed with the gene encoding $HpaG_{xooc}$ protein to know, if there would an additive effect due to the presence of the bacterial gene. The greenhouse experiments showed that the transformant OKBHF was more effective in enhancing plant growth of tomato plants and in lowering the disease severity of *Cucumber mosaic virus* at 28 days post-inoculation (dpi). Plant height, fresh weight and flower and fruit number were greatly increased. The reverse transcription (RT)-PCR analysis revealed the molecular mechanisms of $HPaG_{xooc}$ and *B. subtilis* in tomato plants. The results suggested their synergistic roles in inducing enhanced expression of three expansin genes LeEXP2, LeEXP5 and LeEXP18 which could regulate plant cell growth and two defense-related genes *Pti4* and *Pti6* which could activate expression of a wide array of PR genes and one defense gene PR-1a (Wang et al. 2011).

5.1.2.4 Factors Influencing the Biocontrol Activity

Environmental factors have definite influence on the host plant, bacterial pathogen and the biocontrol agent expected to suppress the development of the pathogen and the disease caused. *Bacillus* spp. are known to produce endospores that are resistant to adverse environmental conditions and these spores remain alive for several years facilitating perpetuation and persistence of these bacteria in the soil and other substrates. The biocontrol agents, in most cases, are confined to the plants/soils on which they are applied. Rarely they are able to spread to other plants in the same field. Natural agencies like wind and insects may aid the biocontrol agents to spread to other plants or locations. Honey bees are important natural pollinators of commercial blueberries in the United States. But they are also the vector of the fungal pathogen *Monilinia vaccinii-corymbosi,* causing mummy berry disease. *Bacillus subtilis* was effective in suppressing flower infection by *M. vaccinii-corymbosi*. Individual honey bees could carry $5.1 \times 6.4 \times 10^5$ CFU of *B. subtilis*, when exiting hive-mounted dispensers with Serenade. On caged rabbiteye blue berry bushes in the field, population densities of *B. subtilis* vectored by honey bees reached a carrying capacity of $<10^3$ CFU per flower stigma within 2 days of exposure. There was a

highly significant non-linear relationship between *B. subtilis* populations per stigma. The bee activity was expressed as number of legitimate flower visits per time interval per cage. The results suggested that the use of a hive-dispersed biocontrol product as a supplement during pollination might reduce the risk of mummy berry disease (Dedej et al. 2004).

The ability of two pollinators *Apis mellifera* and mason bee *Osmia cornuata* was assessed as carriers of the BCAs from flower to flower (secondary colonization on apple cv. Golden Delicious). *Bacillus subtilis* strain BD170 (commercial product Biopro®) was developed for the control of fire blight disease caused by *Erwinia amylovora.* The behavior and capacity of the insects to deposit the BCA in the most receptive flower parts were compared both by washing, diluting and plating the flower organs on a recovery medium and also by applying PCR assays, based on a molecular marker. *O. cornuata* was efficient than *A. mellifera.* Under field conditions, the efficacy of pollinators in carrying the BCA from sprayed flowers to the stigmas of newly opened ones at different intervals after the spray application of the BCA product was determined, by detecting the BCA using PCR assays. The percentages of positive PCR flower samples were higher in the internal treated areas of the fields, compared with external untreated ones. However, the high colonization level found in the external untreated areas and in the flowers opened in both areas at several days after the treatment, indicated that the pollinators could play an important role as secondary carriers (Maccagnani et al. 2009).

Strains of *Bacillus* spp. have been shown to exhibit a desirable trait of eliciting ISR in different plant species against several microbial plant pathogens (Jetiyanon and Kloepper 2002). The individual *B. amyloliquefaciens* strain IN937a, and compatible mixtures of strains of *B. pumilus* strains 937b, SE34, SE49, T4 and INR7 were evaluated for their ability to elicit ISR against Southern blight disease *Sclerotium rolfsii* in tomato, anthracanose disease (*Colletotrichum gloeosporioides*) in long Cayenne pepper (*Capsicum annuum* pv. *acuminatum*) and cucumber mosaic disease (*Cucumber mosaic virus*) in cucumber. The strain mixtures could suppress more consistently both disease incidence and/or severity in two seasons, when compared to individual strain IN937a. Mixture IN937a+IN937b elicited ISR effectively against all diseases under field conditions. Cumulative marketable yields were also increased by some mixtures of BCA strains (Jetiyanon et al. 2003).

5.1.3 Paenibacillus *spp.*

The genus *Paenibacillus*, earlier included under the genus *Bacillus* comprises about 80 species differentiated on the basis of 16S rRNA analysis. They are facultatively aerobic, endospore-forming and low G+C Gram-positive bacilli (Ash et al. 1994; Raza et al. 2008). Several strains of *P. polymyxa* have been reported to effectively suppress the development of plant diseases such as seedling blight, wilt and root rot diseases of cucumber and water-melon caused by *Fusarium oxysporum* and *Pythium* spp. (Dijksterhuis et al. 1999; Yang et al. 2004) and sesame damping-off caused by

Pythium spp. (Ryu et al. 2006). The strains of *Paenibacillus* may have different mechanisms of biocontrol activity against fungal pathogens, causing different economically important plant diseases. In addition to the role as biological control agents of plant diseases, *Paenibacillus* spp. are able to fix atmospheric nitrogen in plant rhizosphere, improving the soil fertility (von der Weid et al. 2002).

5.1.3.1 Antibiosis

Paenibacillus lentimorbus isolate CBCA-2 isolated from pistachio leaves showed significant biocontrol potential against *Botryosphaeria dothidea,* causing pistachio panicle and shoot blight disease. The isolate CBCA-2 inhibited the germination of pycnidiospore entirely after 24 h of incubation at 25 °C. Malformation of pycnidiospores and hyphae, in addition to lysis of the pycnidiospores, was observed. Nutrient yeast dextrose broth induced the secretion of antifungal compounds to the maximum extent, among the five media tested. Development of the lesions on detached leaves was suppressed by application of culture filtrates (CF) of CBCA-2, while the washed bacterial cells did not show any inhibitory effect on lesion development. Application of CF on excised dormant stems, prior to challenge inoculation by the pathogen, was effective in arresting lesion development. Pruning wounds could be effectively protected by spraying a suspension of CBCA-2, prior to inoculation with the pycnidiospore suspension of *B. dothidea* (Chen et al. 2003). Foliar application of *P. lentimorbus* B-30488 reduced the incidence of tomato early blight disease caused by *Alternaria solani* by 45.3 %, compared with control. The bacterial strain reduced the radial growth of *A. solani* in dual culture. Observations under scanning electron microscope (SEM) revealed complete degradation of pathogen hyphae on co-culture with the strain B-30488, indicating direct inhibitory effects of the bacterial BCA and *A. solani*. Gene expression studies, using RT-PCR analyses, indicated that the defense and growth-related genes were up-regulated significantly in tomato plants treated with B-30488 foliar spray and *A. solani*-inoculated plants treated with B-30488 strain. Tomato plants inoculated with *A. solani* affected microbial community structure and population of rhizosphere, as compared with B-30488. The results indicated the multiple modes of action of *P. lentimorbus* B-30488 for its biocontrol activity against *A. solani* through degradation of pathogen cell wall, induction of resistance and possibly by competing for similar sources of nutrients, since the BCA and pathogen exhibited high similarity in nutrient utilization of carbon sources present in tomato tissues (Khan et al. 2012).

Production of fusaricidin-type antibiotics was observed in *Paenibacillus polymyxa* by Beaty and Jensen (2002). *P. polymyxa* 1460 secreted lectins that could enhance cellulose β-glucosidase activity in wheat root cell wall (Karpunia et al. 2003). *Paenibacillus polymyxa* has been reported to produce peptide antibiotics more commonly. *P. polymyxa* E681 produced polymyxin, fusaric acid and polyketides (Ryu et al. 2006). Polymyxin is the main type of peptide antibiotics produced by some strains of *P. polymyxa*, while other strains secreted different peptides including

polypeptins. Polymyxins A, B, C and D have been distinguished, based on the differences in amino acid or fatty acid composition (Orwa et al. 2002). Polymyxins have bactericidal effect on Gram-negative bacilli, especially *Pseudomonas*. Polymyxins alter cytoplasmic membrane permeability by binding to a negatively charged site in the lipopolysaccharide layer (Wiese et al. 1998).

The antagonistic activity of *Paenibacillus brasilensis* strain PB177 against *Fusarium moniliforme* and *Diplodia macrospora* infecting maize was investigated. The antifungal compounds produced by *P. brasilensis* inhibited the mycelial growth of these fungal pathogens and also *Rhizoctonia solani* and *Verticillium dahliae* capable of infecting a wide range of crops. The inhibitory effect of PB177 was stronger on *D. macrospora* than on *F. moniliforme*. Conidial structures of the fungal pathogens were not formed in the presence of PB177, indicating that cellular differentiation of both *D. macrospora* and *F. moniliforme* was inhibited. In the control without bacteria, conidia were produced abundantly (von der Weid et al. 2005). The mechanism of biocontrol activity of *Paenibacillus polymyxa* strains B2, B5 and B6 against *Pythium aphanidermatum* and *Phytophthora palmivora* was investigated using dual culture method on plates containing V8 medium (20 % v/v), consisting of 0.3 %, $CaCO_3$, 1.5 % nutrient agar and 0.003 % cholesterol (w/v, at 28 °C for 48 h in the dark and an additional 48 h under illumination). None of the strains of *P. polymyxa* inhibited the radial growth of *P. aphanidermatum*. In contrast, all strains drastically inhibited the radial growth of *P. palmivora.* However, *P. polymyxa* strains B2 and B5 were able to antagonize the zoospores of both *P. aphanidermatum* and *P. palmivora* around the roots of *Arabidopsis.* The strains B5 and B6 produced similar quantities of antagonistic substances, but the strain B5 was superior in the production of mycoidal substance and biofilm (Timmusk et al. 2009).

Strains of *Paenibacillus polymyxa*, in addition to antibiotics, are able to produce hydrolytic enzymes that play an important role in the biocontrol of plant diseases. *P. polymyxa* 72 produced an amylase (MW 48-kDa) composed of 1,161 amino acids. The gene encoding the biosynthesis of the amylase appeared to be divided into two segments by a direct-repeat sequence located almost at the center of the gene (Sakurai et al. 1989). Two *P. polymyxa* strains could produce cellulase and mananase independent of the presence of monosaccharide products in growth media. Such media-independent expression of enzymes for degradation of cellulose containing cell wall components indicated that these strains could be promising candidates for application as biocontrol agents against fungal pathogens (Nielson and Sørensen 1997). *Paenibacillus polymyxa* strain BRF-1 isolated from soybean rhizosphere was able to suppress the soybean root rot disease caused by a complex of *Fusarium oxysporum* var. *redolens, F. avenaceum, F. solani, Pythium ultimum, Rhizoctonia solani* and *Phytophthora* f.sp. *glycinea*. In a dual culture, the strain BRF-1 inhibited the growth of *R. solani* and *F. oxysporum* as well as other fungal pathogens. The strain BRF-1 produced antifungal peptide. The antifungal compound was separated by ammonium sulfate precipitation and purified by Sephadex G-50. The peptide had a MW of about 35.4-kDa with antifungal activity against *R. solani* (Chen et al. 2010).

5.1.3.2 Colonization of Plant Surfaces

Colonization of plant surfaces by bacterial biocontrol agents prior to initiation of infection by the plant pathogens in specific sites on plant organs is important for successful and effective prevention/inhibition of the development diseases. The importance of bacterial biofilm established on plant roots as a biocontrol mechanism has been emphasized by some investigations. *Paenibacillus polymyxa* could excrete its biofilm formation at the sites of infection by oomycetes as a niche exclusion approach. Microbial biofilms are constituted by both bacterial cells and extracellular matrix which may form upto 98 % of the biofilm (Sutherland 2001; Kolter 2005). Plant root exudates and root electrical signals selectively influence bacterial colonization and biofilm formation. Colonization rarely occurs as individual cells (Van Loon 2007). Efficient root colonization by certain PGPRs is linked to their ability to secrete a site-specific recombinase. Genetically unmodified *P. polymyxa* strains colonize plant root tips as a biofilm. The pattern of biofilm formation has been characterized. The biofilm contains almost infinite range of macromolecules produced by the bacteria.

Paenibacillus brasilensis strain PB177 was tagged with the *gfp* gene encoding the green fluorescent protein (GFP) in order to monitor interactions between PB177 and *Diplodia macrospora* and *Fusarium moniliforme* infecting maize. Bacterial colonization of plant surfaces such as roots and leaves occurs commonly in the form of aggregates or microcolonies. The seeds were treated with GFP-tagged bacteria prior to challenging them with the fungal pathogens on agar plates. Both GFP-tagged PB177 strain and fungal hyphae were detected along with maize root surface in most treated plants. The results indicated that the bacterial cells were mobilized to the maize roots in the presence of the fungal pathogens *D. macrospora* and *F. moniliforme*. The GFP-tagged *P. brasilensis* introduced into the soil could be detected as aggregates attached to plant roots from second day after planting, but colonization patterns were heterogenous and patchy. GFP-tagged PG177 cells introduced into the soil were not commonly observed inside the maize root tissue indicating that *P. brasilensis* could not be considered as an endophyte of maize plants (von der Weid et al. 2005). *Paenibacillus polymyxa* strains B2 and B5 formed biofilms that enlarged throughout 25 h of experimental duration. *P. polymyxa* was found to be effective in protecting the *Arabidopsis thaliana* model system against root colonization by zoospores of the oomycete pathogens *Pythium aphanidermatum* and *Phytophthora palmivora*. The zoospores of *P. palmivora* colonized plant roots in liquid assays and the presence of *P. polymyxa* prevented colonization prior to encystment and infection. The strains of *P. polymyxa* were able to antagonize the zoospores of *P. aphanidermatum* and *P. palmivora* around the *Arabidopsis* root. Most of plants treated with *P. polymyxa* survived the *P. aphanidermatum* inoculations in soil assays. The results indicated that biofilm formation and niche exclusion mechanisms do have a role in the biocontrol activity of bacterial biocontrol agents against plant pathogens (Timmusk et al. 2009).

5.1.3.3 Induction of Resistance to Crop Diseases

The plant growth promoting rhizobacteria (PGPR) *Paenibacillus alvei* strain K-165 was found to be effective against *Verticillium dahliae* causing wilt diseases of several solanaceous crops, including the model plant *Arabidopsis thaliana* (Tjamos et al. 2004). Strain K-165 significantly reduced the development of vascular symptoms in *A. thaliana.* The BCA and the pathogen were spatially separated and hence, antagonism by direct interaction could be ruled out. The strain K-165 induced systemic resistance in *A. thaliana* wild-type (wt) plants against *V. dahliae.* ISR triggered by strain K-165 differed from ISR induced by *P. fluorescens* WCS417r or CHA0. ISR induced by strain K165 was salicylic acid (SA)-dependent, while the resistance induced by *Pseudomonas* strains was entirely independent of SA. ISR induced by strain K-165 against *V. dahliae* was blocked in *Arabidopsis* mutants *sid1/eds 5, sid2* and *npr1-1,* indicating that components of pathway from isochorismate, most likely SA, as well as a functional NPR1 play a crucial role in the ISR induced by the strain K-165. In addition, the concomitant activation and increased transient accumulation of the PR-1, PR-2 and PR5 genes were discernible in the treatment in which both the BCA strain and the challenging pathogen were present in the rhizosphere of *A. thaliana* plants (Tjamos et al. 2005). The biocontrol potential of chitinase-producing *Paenibacillus illinoisensis* strain KJA-424 was assessed against *Phytophthora capsici* infecting pepper (chilli) plants. The growth response of pepper plants and kinetics of pathogenesis-related (PR) protein production were determined after challenge inoculation of *P. capsici* and combined inoculation with the pathogen and the bacterial strain. Root mortality of plants treated with the pathogen and BCA was significantly reduced, compared with pathogen inoculated plants. The activities of β-1,3-glucanase, cellulase and chitinase were significantly increased in plants inoculated with the pathogen and bacterial strain, compared with plants inoculated with the pathogen alone. The activities of these defense-related enzymes were negatively correlated with root mortality. The fresh weight of plants in this treatment was also increased indicating the growth promotion effect of the treatment with *P. illinoisensis* on pepper plants (Jung et al. 2005).

The mechanisms underlying the biocontrol activity of strains of *Paenibacillus polymyxa* MB02-1007 and chitosan against *Ralstonia solanacearum*, causative agent of bacterial wilt diseases of tomato, potato and a large number of plant species, were studied. Most of the strains (14 of 16) did not show any direct inhibitory effect on *R. solanacearum* in dual cultures. On the other hand, chitosan exhibited strong antibacterial activity in paper disk and agar well tests. In the greenhouse assays, chitosan was more effective as soil drench. The effects of treatments with the bacteria and/or chitosan on the enzymatic activities involved in the defense mechanisms operating in the plants were assessed. The chitinase activity was enhanced by both chitosan and *P. polymyxa,* irrespective of method of application (seed treatment/soil drenching) in the presence or absence of the pathogen. *P. polymyxa* increased the chitinase activity as a soil drench or seed treatment by 68 and 78 % respectively, while chitosan increased chitinase activity to a greater extent

(96 %) as soil drench. Changes in the β-1,3-glucanase activity induced by the bacteria and chitosan were less marked, compared with those of chitinase activity. In addition, *P. polymyxa* and chitosan improved the growth of treated tomato plants significantly, demonstrating the beneficial effects of the bacteria and organic compound (Algam et al. 2010).

The ability of lipopolypeptides (paenimyxin) secreted by *Paenibacillus* sp. strain B2 to elicit production of hydrogen peroxide (H_2O_2) and to activate defense-related genes in the model legume *Medicago truncatula* was assessed. A pathosystem between *M. truncatula* cell suspension and *Fusarium acuminatum* was established to investigate the process of induction of resistance to disease. Induction of H_2O_2 reached the peak at 20 min after elicitation with paenimyxin in plant cell cultures. Higher concentration of paenimyxin (>20 μM) inhibited the induction of H_2O_2. In plant roots, paenimyxin at a low concentration (1 μM) applied prior to inoculation with *F. acuminatum* showed protective effect and suppressed 95 % of necrotic symptoms. Inhibitory effect of paenimycxin was observed at higher concentration on plant growth. A semi-quantitative reverse transcription (RT)-PCR assay was applied to quantify the gene responses in *M. truncatula*. Genes involved in the biosynthesis of phytoalexins (enzymes phenylalanine ammonia lyase, chalcone synthase and chalcone reductase), antifungal activity (PR-proteins and chitinase) or cell wall (invertase) were highly upregulated in roots or cells after paenimyxin treatment (Selin et al. 2010).

5.1.4 Burkholderia *spp.*

Burkholderia cepacia as a plant pathogen was first reported on onion bulbs by Burkholder (1950). The bacterial species was later found to be a ubiquitous soil bacterial species and has emerged as an important biocontrol agent of plant pathogens. *Burkholderia cepacia* complex (*Bcc*) is naturally abundant in soil, water, on plant surfaces and in clinics. The *Bcc* consists of nine discrete genomic species and a genetic scheme based on the *recA* gene facilitated greatly the identification of *B. cepacia* complex species. This molecular approach was useful to differentiate the pathogenic and nonpathogenic human forms and nine current species within the genus *Burkholderia* (Mahenthiralingam et al. 2000). Different strain of *B. cepacia* were shown to be effective against *Pythium*-induced damping-off disease (Parke et al. 1991), *Aphanomyces*-induced root rot disease of pea (King and Parke 1993) and *Rhizoctonia*-induced root rot of poinsettia (Cartwright and Benson 1994). The effectiveness of *B. cepacia* against the bacterial pathogen *Ralstonia solanacearum* causing tomato bacterial wilt disease was also demonstrated (Sfalanga et al. 1999). The use of *B. cepacia* as a biocontrol agent of phytopathogens became a point of great concern, because of the report providing evidence for its ability to cause cystic fibrosis in human beings suffering from respiratory ailments (Isles et al. 1984).

Burkholderia cepacia produces antimicrobial compounds inhibiting the development of plant pathogens. *B. cepacia* AMMD effectively suppressed the development

of Pythium damping-off and Aphanomyces root rot disease of peas, when applied as seed treatment in growth chamber and field experiments (Parke et al. 1991). The antagonistic effects of *B. cepacia* strain AMMDR1 on post-infection stages in the life cycles of *Pythium aphanidermatum* and *Aphanomyces euteiches* infecting peas were assessed. The total numbers of oogonia per root were reduced by approximately 60 % at 12 days after inoculation with zoospores in treatments where *B. cepacia* AMMDR1 strain was applied, independent of whether the bacterial strain was applied to seeds or roots. In contrast, treatment with *B. cepacia* strain 1324 did not exert any adverse effect on oogonial production by the pathogens. Cross-sections of the root tissues in the area of zoospore inoculation showed that the reduction in the number of oogonia formed by the strain AMMDR1 was most dramatic in the cortex and much less in the stele. This situation may suggest that the antibiotics produced by the BCA may diffuse with difficulty across the endodermis. The bacterial strain had no effect on the production of secondary zoospores of *A. euteiches* from infected pea roots. The results indicated that all effects of *B. cepacia* strain AMMDR1 were dependent on the application or in situ production of antibiotics at the site of pathogen activity. *B. cepacia* could restrict the seed infection by *P. aphanidermatum* and reduce the mycelial growth and production of oogonia by *A. euteiches* (Heungens and Parke 2001). *Burkholderia cepacia* strain AMMDR1 was able to reduce effectively the severity of damping-off disease caused by *Pythium aphanidermatum* and root rot disease caused by *Aphanomyces euteiches* in peas. In order to investigate the underlying mechanisms of biocontrol activity of the strain AMMDR1, in addition to antibiosis, an antibiosis deficient strain was employed. *B. cepacia* strain 1324, a seed and root colonizing Tn5 mutant of the strain AMMDR1 was evaluated for its biocontrol potential in suppressing the development of pea damping-off and root rot diseases, along with the wild-type strain. The seedling emergence was substantially increased from 0 to 46 %, when seedlings were treated with strain AMMDR1 at 4 h after inoculation with *P. aphanidermatum.* On the other hand, the mutant 1324 did not show any effect on seedling emergence. The wild-type strain significantly reduced post-infection colonization of pathogen and damping-off of pea seedlings, even when the bacteria were applied at 12 h after zoospore inoculation. The mutant strain had no effect on mycelial colonization of seeds or roots by *A. euteiches* also, suggesting that the primary mechanism of biological control by *B. cepacia* AMMDR1 was through antibiosis (Heungens and Parke 2001).

Two isolates of *Burkholderia cepacia* BC-S and BC-TM were evaluated for their potential as biocontrol agents against *Schizophyllum commune* causing seed rot of oil palm and *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) causing wilt disease of tomato. Pathogenicity tests of the two isolates of *B. cepacia* on onion bulbs showed that they induced non-soft rot symptoms. Sunken dried lesions appeared on the outside surface of bulbs around the site of inoculation. Dual culture tests showed that both isolates of *B. cepacia* inhibited the mycelial growth of both pathogens to different degrees. The isolate BC-S was more inhibitory to *S. commune,* while BC-TM was able to inhibit *Fol* more effectively. In addition, these two BCA strains could inhibit the mycelial growth of other fungal pathogens *Fusarium solani* and

Colletotrichum dematium (Sijam and Dikin 2005). A novel compound designated CF661 with antifungal activity was isolated from *B. cepacia* strain CF661. This compound was inhibitory to several fungal pathogens such as *Alternaria alternata*, *Bipolaris sorokiniana, Colletotrichum lindemuthianum, Rhizoctonia solani, Fusarium graminearum* with varying minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). No visible fungal growth could be observed at CF661 concentrate above 20.0 μg/ml. Microscopic observations revealed complete inhibition of conidia with concomitant protoplasm aggregation and broken mycelia in *Fusarium graminearum*, *F. oxysporum* and *Curvularia lunata* treated with CF661 (Li et al. 2007). In the further study, the antifungal effects of CF661 from *B. cepacia* were studied by dual staining with propidium iodide (PI) and fluorescein diacetate (FDA) on *Fusarium solani*. Incubation with 120.0 μg/ml of CF661 showed collapsed hyphae with visualizations of PI and FDA straining, indicating extensive cell death and membrane permeation. High doses of CF661 might kill *F. solani* by acting primarily on cell membrane. At lower concentration (20.0 μg/ml) of CF661, the growth of *F. solani* was entirely inhibited with marked morphological changes in the hyphae which exhibited swelling and numerous balloon-shaped cells. In addition, abnormal chitin deposition in hyphae was also seen (Li et al. 2009).

In cocultures, *B. cepacia* strain Lu 10–1 inhibited the mycelial growth of *Colletotrihum dematium,* causing mulberry anthracnose disease. Hyphae growing close to the bacterial colonies showed excessive branching, irregular swelling, curling of hyphal tips and disruption of apical growth. Coagulation of cytoplasm, degradation of the mycelium and presence of large vesicles inside fungal cell walls were also discernible. Inhibition of conidial germination by cell-free culture filtrate of Lu10-1 indicated the direct antagonistic effects of this bacterial strain (Ji et al. 2010). *B. cepacia* TIA-2B effectively suppressed the mycelial growth of *Rhizoctonia solani* and *Sclerotium rolfsii* infecting tomato. The roots were treated with the bacterial strain. Observations under SEM showed that the isolate TIA-2B could adhere to tomato roots producing biofilm structures at 48 h after application. High colonization density was observed in the apical root zone (De Curtis et al. 2010).

Burkholderia cepacia strain Lu 10–1, was able to suppress the development of the mulberry anthracnose disease caused by *Colletotrichum dematium* through multiple mechanisms of biocontrol activity. In addition to the production of antifungal compound, the strain Lu 10–1 produced siderophores, indole acetic acid (IAA) and solubilized phosphates and exhibited nitrogenase activity, indicating its ability to compete with the pathogen for nutrients and to promote the growth of mulberry plants. The strain Lu10-1 could reduce the disease severity significantly, when applied to inoculated leaves or to the soil, but the extent of disease suppression varied with time interval between pathogen inoculation and bacterial treatment. Presence of green fluorescent protein (GFP)-labeled Lu10-1 cells was monitored in the plants, after application of the labeled bacteria to the soil. The labeled cells were detected mainly in the intercellular spaces of roots and stems. The bacterial cells were found in the xylem vessels and in leaf veins at 11 and 20 days after inoculation respectively, indicating the ability of bacterial cells to migrate from the site of application

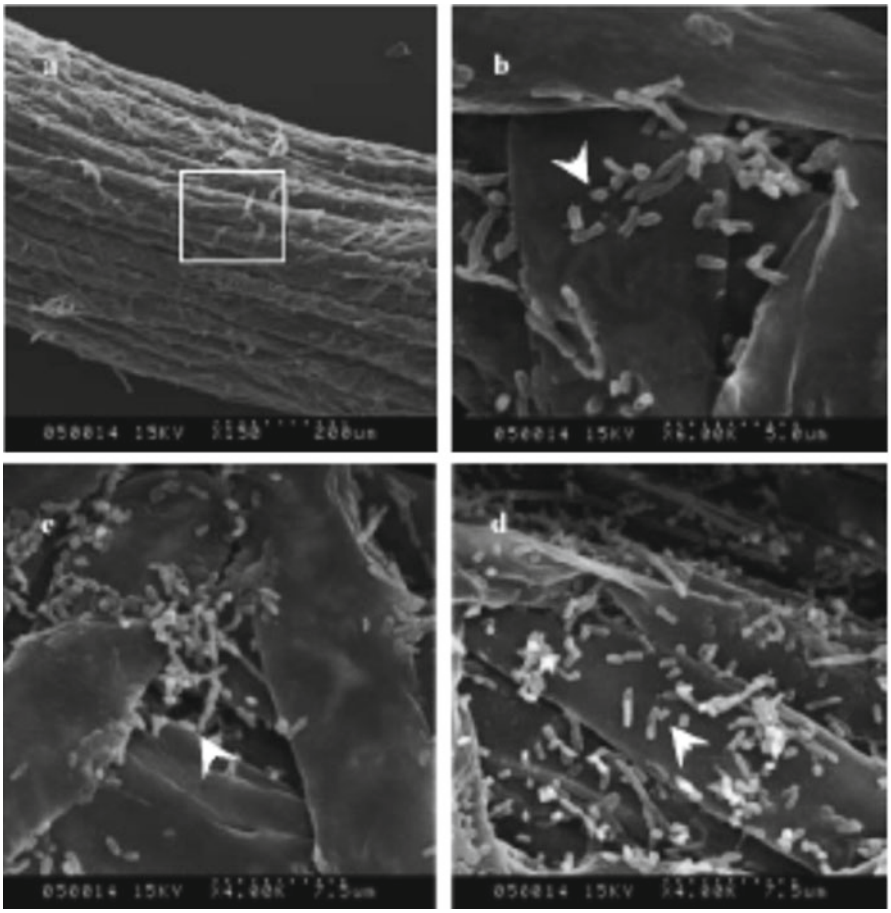

Fig. 5.9 Visualization of colonization of mulberry roots by *Burkholderia cepacia* strain Lu 10–1. (**a**): colonization of root hair zone; (**b**): magnified image of the framed region; (**c**): colonization of sites of root hair emergence; (**d**): colonization of the surface of the root hair (Courtesy of Ji et al. 2010 and with kind permission of BioMed Central, BMC Plant Biology)

(Fig. 5.9). Observations under SEM confirmed that the bacterial cells congregated at many entry sites along the length of the roots. Some bacterial cells were able to enter the cortex directly through the epidermis. The junctions between the primary and secondary roots were heavily colonized, indicating that the bacteria could enter the roots through the fissures or cracks present at the site of emergence of lateral roots and of the radicle. The treated plants showed resistance to anthracnose disease at sites away from the site of Lu10-1 application. The results indicated that the strain Lu10-1 might suppress development of anthracnose disease through direct antagonism, competition for nutrients, as well as by induction of disease resistance (Ji et al. 2010).

5.1.5 Lysobacter

These bacteria formerly placed within the order Myxobacteriales and Cytophagales were transferred to the genus *Lysobacter* that encompases nonfruiting gliding bacteria with a high G+C ratio. *Lysobacter* included *L. antibioticus, L. brunescens, L. enzymogenes* and *L. gummosus* and several strain earlier identified as *Stenotrophmonas* spp. or *Xanthomonas* spp. have been included in the genus *Lysobacter* based on the analysis of 16S rRNA gene sequences and fatty acid composition (Sullivan et al. 2003). Several strains of *Lysobacter* spp. have been reported to be effective biocontrol agents of plant pathogens. The strains of *Lysobacter* spp. produce antibiotics and lytic enzymes that can suppress the development of fungal pathogens. The ability of *L. enzymogenes* strain C3 to induce systemic resistance in treated plants against fungal pathogen has also been demonstrated (Kilic-Ekicio and Yuen 2003). Three strains of *Lysobacter enzymogenes* have been described as biocontrol agents of plant pathogens. Strain 3.IT8 was antagonistic to fungal and oomycetous pathogens, including various species of *Pythium*. Strain N4-7 was found to be effective in suppressing the development of summer patch disease of turfgrass caused by *Magnaporthe poae* (Islam et al. 2005). *Lysobacter enzymogenes* strain C3 was found to be natural antagonist capable of suppressing diseases caused by *Rhizoctonia solani* and *Bipolaris sorokiniana* on turf grass (tall fescue) (Zhang and Yuen 1999; Yuen and Zhang 2001). *L. enzymogenes* isolated from rockwool, effectively suppressed damping-off disease of cucumber caused by *Pythium aphanidermatum*. Another strain *L. capsici* isolated from the rhizosphere of pepper (chilli) plants exhibited antimicrobial activity (Park et al. 2008).

Lysobacter sp. strain SB-K88 suppressed the development of damping-off disease of sugar beet caused by *Pythium* sp. The metabolites inhibiting the mycelial growth were identified as xanthobaccins A, B and C. Seed bacterization and seed treatment with isolated xanthobaccin A suppressed sugarbeet damping-off disease in sugar beet plants grown in *Pythium* sp. infested soil (Nakayama et al. 1999). In a later investigation the modes of biocontrol activity of *Lysobacter* spp. strain SB-K88 against *Aphanomyces cochlioides,* causative agent of damping-off disease of sugar beet were studied. Microscopic observation of *A. cochlioides* hyphae growing close to the bacterial colonies showed excessive branching, irregular swelling, curling of hyphal tips and loss of apical growth. SEM observations revealed dense colonization of the bacteria on the root surfaces and a characteristic perpendicular pattern of *Lysobacter* colonization, possibly generated via development of polar, brush-like fimbrae. The strain SB-K88 was able to colonize also the roots of several plants, including spinach, tomato and amaranthus. Plants growing from the seeds of sugar beet and spinach treated with strain SB-K88 exhibited resistance to the damping-off disease induced by *A. cochlioides.* Exposure of zoospores of *A. cochlioides* to the bacterial cell suspension, cell-free supernatant of SB-K88 or pure xanthobaccin A for a minute, resulted in immobilization of zoospores. In all treatments lysis followed within 30 min in the presence of the inhibiting factors. The results indicated that a combination of antibiosis and characteristic biofilm formation at the rhizoplane

of the host plant might be the mechanism of biocontrol activity of *Lysobacter* sp. strain SB-K88, resulting in effective suppression of damping-off disease of sugar beet (Islam et al. 2005).

Lysobacter enzymogenes strain C3 was shown to be effective against *Bipolaris sorokiniana,* causing Bipolaris leaf spot in turf grasses and *Fusarium graminearum*, causative agent of Fusarium head blight disease of wheat. A heat-stable antifungal factor (HASF) was isolated from the culture supernatant. HSAF was an antibiotic complex consisting of dihydromaltophilin and structurally related to macrocyclic lactams which exhibited suppressive effect on several fungal and oomycete pathogens such as *Bipolaris sorokiniana, Fusarium graminearum, Rhizoctonia solani, Sclerotinia sclerotiorum, Pythium ultimum* var. *ultimum* and *Phytophthora sojae*. When applied to tall fescue leaves as a partially-purified extract, HASF (>25 μg/ml) inhibited germination of *B. sorokiniana* conidia. Two mutants of the strain C3 disrupted in the hybrid polyketide synthase-nonribosomal peptide synthetase gene for HSAF biosynthesis were less efficient in suppressing the development of Bipolaris leaf spot, compared with the wild type strain due to their in ability to synthesize HSAF. In contrast, the biocontrol potential of the mutants in reducing the severity of Fusarium head blight was in equivalence with the wild-type strain. The role of HSAF was not important for the strain C3 as a biocontrol agent against all fungal pathogens, since the HSAF-minus mutants were equally effective as the wild-type against diseases like Fusarium head blight of wheat (Li et al. 2008a, b). *Lysobacter enzymogenes* strain 3-IT8 was effective in suppressing the development of root and crown rot disease caused by *Pythium aphanidermatum* in rockwool hydroponic system. Application of the strain 3-IT8 in combination with chtiosan (the deacetylated derivative of chitin) reduced the number of diseased plants by 50–100 %, compared with control. Application of chitin alone or the bacterial strain alone was ineffective. The chitosan disappeared from the hydroponic system within 24 h after application which could be attributed to the expression of enzymes of the strain 3-IT8 induced by the exposure to chitosan. The results indicated that addition of chitosan enhanced the biocontrol efficacy of *L. enzymogenes* and chitosan might serve as C- and N-source for the bacterial strain, and induce antagonist gene expression or both (Postma et al. 2009).

Lysobacter enzymogenes produces abundantly extracellular enzymes such as chitinases and glucanases that can degrade the major cell wall components of fungi and oomycetes. The role of extracellular enzymes in the biocontrol activity of *L. enzymogenes* strain C3 has been studied. Purified chitinolytic fractions inhibited the spore germination and infection of plants by *Bipolaris sorokiniana* (Zhang and Yuen 2000). *Lysobacter enzymogenes* strain C3 produced chitinases in broth media containing chitin. The conditions for production of chitinases by the strain C3 were studied and the partially purified chitinases were characterized after purification by ammonium sulfate precipitation and chitin affinity chromatography. The partially purified fraction inhibited conidial germination and germ tube elongation of *Bipolaris sorokiniana*. The fraction exhibited strong exochitinase and weak endochitinase activity. The activity was maximum at 45–50 °C and pH 4.5–5.0 and Hg^{2+} and Fe^{3+} ions inhibited the activity. Of the five proteins separated from partially purified preparations, three

proteins showed homology to known bacterial chitinases. At least two chitinases produced by the strain C3 were found to have antifungal properties (Zhang et al. 2001). *L. enzymogenes* strain 34S1 was found to be an effective biocontrol agent against *Magnaporthe poae* causing summer patch disease of Kentucky bluegrass (*Poa pratensis*). A chitinase gene was cloned on a 2.8-kb DNA fragment from the strain 34S1 by heterologous expression in *Burkholderia cepacia*. A 51-kDa protein with chitinolytic activity was purified from the culture filtrates of the BCA strain by hydrophobic interaction chromatography. The mutant strain C5 defective in the function of *chiA* gene lacked chitinolytic activity and it did not secrete the 51-kDa protein in culture filtrates. The strain C5 showed decreased ability to suppress summer patch disease on Kentucky grass, indicating the important role of chitinolytic enzyme in the biocontrol activity of *L. enzymogenes* strain 34S1 (Kobayashi et al. 2002).

Lysobacter enzymogenes strain C3 produced multiple extracellular hydrolytic enzymes which were involved in the biocontrol activity of this strain. The regulation of biosynthesis of these enzymes was investigated by generating mutants of strain C3 disrupted in extracellular enzyme production and evaluating the efficiency of the biocontrol activity of the mutants. The *clp* gene encoding a global regulator was found to govern the lytic enzyme production and a number of other factors also in the strain C3. A single min-Tn5-*lacA*,-cat transposon mutant 5E4 of the strain C3 that was globally affected in a variety of phenotypes was isolated. The activities of several extracellular lytic enzymes, gliding motility and in vitro antimicrobial activity of the mutant 5E4 were reduced. The *clp* gene was chromosomally inserted into the mutant 5E4, resulting in complemented strain P1. All mutant phenotypes were restored in P1. But the gliding motility was found to be excessive, compared with that of the wild-type strain. The *clp* mutants had reduced biocontrol efficiency against sugar beet damping-off and tall fescue Bipolaris leaf spot diseases. The biocontrol efficiency was partially restored in the complement strain P1 (Kobayashi et al. 2005). A new isolate *Lysobacter* sp. was obtained from the rhizosphere of tobacco plant and it was identified as *L. capsici* based on the sequencing and phylogenetic analysis of 16S rRNA and characteristics in the Biolog Identification System. The strain PG4 of *L. capsici* exhibited chitinase and protease activity, capable of digesting yeast cells. No siderophore production could be detected on CAS agar plates. The strain PG4 was highly antagonistic to *Rhizoctonia solani* and *Sclerotinia sclerotiorum* and several other fungal pathogens. Seed treatment with the strain PG4 drastically reduced the plant mortality caused by *F. oxysporum* f.sp. *radicis-lycopersici* in the greenhouse experiments (Puopolo et al. 2010).

Lysobacter enzymogenes strain N4-7 produced three β-1,3-glucanases, and the genes *gluA* and *gluC* encoded the biosynthesis of these enzymes. The molecular and biochemical characterization of three enzymes in strain N4-7 provided the basis to assess the role of β-1,3-glucanases in biocontrol activity of the bacterial strains (Palumbo et al. 2003). The strains C3 and N4-7 are closely related. The strain C3, unlike N4-7, is genetically tractable and it has been studied as a biocontrol agent against many pathogens. *L. enzymogenes* strain C3 produced multiple extracellular β-1,3-glucanases encoded by the genes *gluA*, *gluB* and *gluC*. These glucanases of strain C3 resolved in native gels produced patterns nearly identical to those produced by strain N4-7, sharing >95 % amino acid sequence identity to their

Table 5.9 Effect of treatment of wheat and tall fescue with *L. enzymogenes* strain C3 on incidence of severity of diseases (Kilic-Ekicio and Yuen 2003)

Treatments	Bipolaris leaf spot		Rhizoctonia leaf blight area (%)
	Incidence (lesions/10 cm of leaf)	Severity (% of leaf area infected)	
Live C3 cells	4b	1b	20b
Killed C3 cells	6b	2b	35b
Distilled water	41a	13a	74a

In the same column figures followed by the same letter are not significantly different (P = 0.05)

counterparts. Mutational analysis indicated that the three genes accounted for the total β-1,3-glucanases activity detected in culture. Using gene-specific mutagenesis approach, direct evidence was obtained supporting a role for β-1,3-glucanases in the biocontrol activity of the strain C3 against *Bipolaris sorokiniana* and *Pythium ultimum*. The strain G123, mutated in all three glucanase genes, showed significantly reduced biocontrol efficiency against the Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet. The results revealed the important role of the β-1,3-glucanases produced by the strain C3 in its biocontrol activity against fungal pathogens (Palumbo et al. 2005).

Induced systemic resistance (ISR) is one of the important mechanisms of biocontrol activity of rhizobacteria against microbial plant pathogens. The chitinase and glucanase activities of *Lysobacter enzymogenes* strain C3 could not account for all its disease suppressive activity. The spatial distribution of strain C3 on host plant shoots suggested the possibility of induction of resistance to diseases being a possible mechanism of biocontrol activity. Resistance elicited by the strain C3 suppressed germination of *Bipolaris sorokiniana* conidia on the phylloplane of tall fescue, in addition to reducing the severity of leaf spot. Direct pathogen-inhibitory effect could be separated from antibiosis by applying heat inactivated cells of the strain C3 that retained no antifungal activity. Localized resistance occurred, when the leaves were treated with live or heat-killed cells, whereas systemic resistance was induced, when the roots of tall fescue plants were treated. The resistance induced by foliar or root treatments persisted for 15 days. In addition, the dose-relationship typical of antagonism of the BCA was absent, indicating the ability of strain C3 to elicit ISR. The resistance induced by strain C3 was not host or pathogen-specific, since the foliar application of heat-killed cells of C3 suppressed the development of *B. sorokiniana* on wheat and reduced the severity of brown patch caused by *R. solani* in tall fescue (Table 5.9) (Kilic-Ekicio and Yuen 2003). The ability of *Lysobacter enzymogenes* strain C3 to elicit induced resistance in wheat against *Fusarium graminearum* (= *Gibberella zeae*) causing Fusarium head blight (FHB) disease was assessed in the greenhouse. Chitin broth cultures of the strain C3 reduced FHB severity to <10 % infected spikelets, compared to >80 % severity in control treatment. The C3 broth cultures, heated to inactivate cells and lytic enzymes, but retaining the elicitor factor for induced resistance, also effectively reduced FHB

severity, suggesting induced resistance is one mechanism of biocontrol activity. Protection by treatment with C3 was not systemic, since application of the bacterial strain uniformly to cover all susceptible florets was required (Jochum et al. 2006).

5.1.6 Serratia *spp.*

The genus *Serratia* includes *S. liquefaciens, S. marcescens, S. plymuthica* and *S. rubidaea. S. marcescens* and *S. liquefaciens* are frequently found associated with human infections as opportunists. *S. plymuthica* has also been recovered from body, but it has rarely been isolated as the sole bacterial species present. *S. plymuthica* is classified as a member of risk group1. No strong evidence of causing human infections has been obtained (de Vleeschauwer and Höfte 2007). However, *S. plymuthica* has been found in the rhizosphere, as endophyte of plants and it has been demonstrated to be a biocontrol agent that can suppress the development of many soilborne and airborne plant pathogens. Some of the plant pathogens that have been reported to be controlled by strains of *S. plymuthica* include *Rhizocto solani* infecting cotton and bean (Chernin et al. 1995; Ovadis et al. 2004), *Pythium aphanidermatum* infecting cucumber (Ovadis et al. 2004). *Colletotrichum lindemuthianum* and *Botrytis cinerea* infecting bean (Meziane et al. 2006a, b), *Verticillium dahliae* and *Phytophthora cactorum* infecting strawberry (Berg et al. 2001) and *Phytophthora capsici* infecting pepper (chilli) (Park and Shen 2002; Shen et al. 2002). The effectiveness of *S. marcescens* as a biocontrol agent against *Phytophthora parasitica* causing citrus gummosis disease was reported by de Queiroz and de Melo (2006). Strains (16) of *Serratia plymuthica* showed antifungal activity against *Verticillium dahliae, Rhizoctonia solani* and *Sclerotinia sclerotiorum* in in vitro bioassays. Dipping strawberry roots in a suspension of cells of *S. plymuthica* strain HRO-C48 reduced the Verticillium wilt disease incidence by 18.5 % and root rot disease caused by *Phytophthroa cactorum* by 33.5 % (Kurze et al. 2001). *S. plymuthica* strain A21-4 isolated from the rhizosphere soils and onion roots, colonized the roots of pepper, when it was applied to the seeds or roots of pepper. It was inhibitory to *Pythium* sp. and *Phytophthora capsici*, but not to *Fusarium* sp. and *Rhizoctonia solani*. The strain A21-4 population on the rhizosphere remained at high levels (up to 10^6 CFU/g soil) after a period of 21 days (Shen et al. 2002). *Serratia marcescens* strain R-35 was isolated from the roots of healthy citrus plants. In plate confrontation tests, the strain R-35 was able to inhibit the mycelial growth of *Phytophthora parasitica* causing citrus gummosis disease. The bacterial strain lysed the oospores of *P. parasitica*. The antagonistic activity of the strain R-35 was considered to be due to antibiotics as well as siderophores produced by the BCA. Application of the strain R-35 reduced the citrus seedling infection by 50 % (de Queiroz and de Melo 2006).

S. plymuthica strain A153 suppressed the production of apothecia by *Sclerotinia sclerotiorum* and the inhibitory effect was attributed to the production of chlorinated macrolides (Thaning et al. 2001; Levenfors et al. 2004). In addition, production of

antibiotics such as pyrrolnitrin (Prn), prodigiosin and haterumalides by *S. plymuthica* has also been reported (Chernin et al. 1996; Thaning et al. 2001; Levenfors et al. 2004). Isolated haterumalides, purified from the supernatant of *S. plymuthica* strain A153 drastically inhibited the mycelial growth, apothecial formation and ascospore germination of many filamentous fungi and oomycetes in vitro (Strobel et al. 1999; Levenfors et al. 2004). *S. plymuthica* IC1270 was found to be effective against *Penicillium expansum,* causing apple blue mold disease and *Monilinia fructicola* infecting peaches. Pyrrolnitrin was considered to be the key factor in suppressing the postharvest diseases of apples and peaches (Rite et al. 2002). Gene expression and regulation of biosynthetic genes for Prn in *S. plymuthica* strain IC1270 was studied. The Prn-deficient *grrA, grrS* and *rpoS* gene replacement mutants were less efficient in suppressing the development of *R. solani* and *P. aphanidermatum* in greenhouse assays, indicating that the biocontrol activity of this strain was tightly modulated by the GrrA/GrrS global regulatory cascade and the sigma factor RpoS (Ovadis et al. 2004). The soilborne pathogen *Pythium ultimum* causes damping-off disease in greenhouse and field grown cucumber. Live cells and ethanol extracts of cultures of *Serratia marcescens* strain N4-5 significantly suppressed the development of damping-off disease, when applied as seed treatment for cucumber seeds. Culture filtrates of N4-5 exhibited chitinase and protease activities, whereas the ethanol extracts contained the antibiotic prodigiosin, the surfactant serrawettin W1 and possibly other unidentified surfactants. Production of prodigiosin and serrawettin W1 was temperature dependent, since their production occurred at 28 °C, but not at 37 °C. Prodigiosin purified from two consecutive TLC runs, using different solvent systems, inhibited germination of sporangia and mycelial growth of *P. ultimum.* The surfactant serrawettin did not have any inhibitory effect on the pathogen. Disease suppression by *S. marcescens* strain N4-5 was in part due to the production of the antibiotic prodigiosin (Roberts et al. 2007).

Biocontrol agents parasitic on fungal pathogens excrete extracellular cell wall-degrading enzymes such as chitinases, glucanases and proteases that target pathogen cell wall, resulting in lysis of the pathogen cells. The biocontrol potential of *Serratia marcescens, Streptomyces viridodiasticus* and *Micromonospora carbonacea* against *Sclerotinia minor* causing basal drop disease of lettuce was evaluated. These bacteria and actinomycete effectively suppressed the pathogen development and disease incidence. All the three BCAs were efficient producers of chitinase and β-1,3-glucanase and caused extensive plasmolysis and cell wall lysis of *S. minor*. Production of chitinases and β-1,3-glucanases was considered to be the main mechanism of biocontrol activity of these BCAs, since only the production of these enzymes was related to the extent of in vitro pathogen inhibition and disease suppression. The transfer of the plugs containing *S. minor* inhibited by the antifungal enzymes, to a fresh medium and their inability to grow further, indicated a fungicidal effect of the metabolites produced by the BCA isolates. When a mixture of all three antagonists was applied to soil in glasshouse trials, disease incidence was not significantly different from the most effective strain *S. marcescens*. This indicated that there was no additive effect on disease incidence due to the presence of the actinomycetes. In addition, *S.*

marcescens was the most competent rhizosphere colonizer and reduced the incidence of basal drop disease caused by *S. minor* (El-Tarabily et al. 2000). Different types of chitinases were produced by the strains of *S. plymuthica*. A chitinase-encoding gene *chiA* from *S. plymuthica* strain IC1270 was cloned and sequenced. The *chiA* gene had an open reading frame coding for 562 amino acids of a 61-kDa precursor protein with a putative leader peptide at the N-terminus. *Escherichia coli* transformed with *S. plymuthica chiA* gene was able to suppress the development of *R. solani* and inhibit spore germination of *F. oxysporum* f.sp. *melonis* in vitro. In addition, the transformant suppressed root rot disease development in cotton seedlings under greenhouse conditions (Chernin et al. 1997). The role of chitinases in the biocontrol activity of *S. plymuthica* strain IC14 against *S. sclerotiorum* and *B. cinerea* appeared to be less important. Synthesis of proteases and other biocontrol traits were required for the suppression of *B. cinerea* and *S. sclerotiorum* by this strain (Kamensky et al. 2003). Biocontrol activity of the strain IC1270 against *R. solani* and *P. aphanidermatum* was demonstrated to be, not dependent on the production of chitinase by this strain (Gavriel et al. 2004). Biocontrol activity dependent on chitinolytic enzymes was variable and strain-specific.

Competition for nutrients among microorganisms especially for bioavailable iron in soil habitats and on plant surfaces, under iron-limiting conditions, is well known. Strains of *Serratia* may produce a range of low-molecular weight compounds or siderophores to sequester iron compounds, thereby depriving the fungal pathogens of this element required for their growth. *S. plymuthica* strains IC1270, IC14, and HRO-C48 were shown to produce potent siderophores functioning as iron-chelators in vitro under iron-limited conditions (Kamensky et al. 2003; Berg et al. 2005; Ovadis et al. 2004; Faltin et al. 2004). The Prn- and/or – endochitinase mutants of the strain IC1270 exhibited residual biocontrol activity. Despite the loss of ability to secrete the antibiotic and enzyme considered to have a role in the biocontrol activity of these mutants was suggested to be due to their ability to compete for nutrients like iron (Gavriel et al. 2004; Ovadis et al. 2004). The mechanism of biocontrol activity of two strains IC1270 and IC14 of *Serratia plymuthica* against *Penicillium digitatum* (orange green mold) or *P. italicum* (blue mold of orange) was investigated. Two mutants of strain IC1270, one deficient in chitinolytic activity (IC1270-C7) and another deficient in pyrrolnitrin production (IC1270-P1) were generated by gene replacement technique. On orange, the mutants were as effective as wild-type strain, suppressing the development of *P. digitatum.* However, the loss of antifungal activity by IC1270-P1 in the in vitro assays was observed, as no inhibition zone could be recognized, when tested against *P. digitatum* or *P. italicum*. The chitinase-deficient mutant of strain IC14 was found to be equally efficient as the wild-type strain, suggesting that chitianses did not have any role in the suppression of green mold disease caused by *P. digitatum*. Interactions between strains IC1270 or IC14 and *P. digitatum* were investigated, using tissue culture plates with diluted orange peel extract as the nutrient source. Strain IC1270 decreased germination of *P. digitum* conidia, when it was physically separated from the pathogen by a membrane filter, which permitted nutrient and metabolite interchange, while strain IC14 did not inhibit spore

germination. But significant inhibition of conidial germination of *P. digitatum* was recorded, when the pathogen and IC14 were in physical contact with each other. Competition for nutrient appeared to be the principal mechanism of strain IC1270, while a direct cell to-cell interaction between IC14 and the pathogen was required for antagonistic activity (Meziane et al. 2006a, b).

Enhancement of levels of resistance of host plant by rhizobacteria is well known. Some strains of *Serratia plymuthica* have been reported to elicit induced systemic resistance (ISR) against plant pathogens. *S. plymuthica* strain 2–67 induced systemic resistance in cucumber against the anthracnose pathogen *Colletotrichum orbiculare* (Gang et al. 1991). Elicitation of ISR in cucumber against *Pythium ultimum* by *S. plymuthica* strain RICG4 was also observed. The structural and biochemical changes in cucumber treated with the strain RICG4 were studied using electron microscopy. ISR, elicited by the bacterial strain, correlated with the formation of structural barriers that might prevent pathogen ingress towards the vascular stele. This was accompanied by the deposition of a phenolic enriched occluding material. The responses linked to the development of ISR included oxidation and polymerization of pre-existing phenols and synthesis of new phenolic compounds via activation of the phenyl-propanoid pathway. By priming the susceptible cucumber plants using suspension of the strain R1CG4, the development of root rot disease caused by *P. ultimum* could be suppressed (Benhamou et al. 2000). The strain IC1270 could elicit ISR against *Botrytis cinerea* and *Colletotrichum lindemuthianum* in bean and tomato (Meziane et al. 2006a, b). *Serratia plymuthica* HRO-C48 isolated from the rhizosphere of oilseed rape produced *N*-acyl homoserine lactone (AHL) signals which mediated quorum sensing regulation. The strain HRO-C48 produced the broad-spectrum antibiotic pyrrolnitrin. This strain induced systemic resistance against *Botrytis cinerea* causing gray mold disease in bean and tomato plants. Colonization of the rhizosphere depended on AHL signaling. The results indicated that quorum sensing regulation might be generally involved in interactions between plant-associated bacteria, fungal pathogens and host plants (Pang et al. 2009).

The differential effects of the strain IC1270 against rice pathogen have been reported. This bacterial strain induced systemic resistance against the blast disease caused by *Magnaporthe grisea* and bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In contrast, the infection by the necrotrophic pathogens *Rhizoctonia solani* causing sheath blight disease and *Bipolaris oryzae* causing brown spot disease was significantly promoted. The differential effectiveness of IC1270 may possibly be due to its capacity to modulate oxidative machinery of the rice plant (de Vleeschauwer and Höfte 2007). The root colonization by IC 1270 in rice did not cause a strong constitutive resistance phenotype, but rather primed plants to hyper-respond to subsequently inoculated plants, resulting in excessive defense activation and enhanced resistance to *Magnaporthe oryzae*. This priming effect of IC1270 was revealed by the observation that challenge inoculation of IC1270-colonized plants with *M. grisea* entailed a rapid accumulation of autofluorogenic phenolic compounds in and around epidermal cells displaying dense cytoplasmic granulation (Fig. 5.10). The IC1270-inducible ISR response seemed to get as a double-edged sword within the rice defense

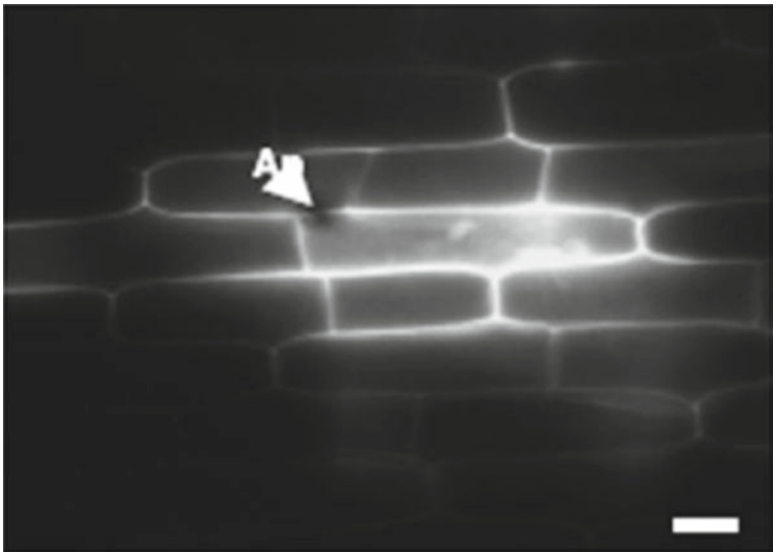

Fig. 5.10 Accumulation of autofluorescent phenolics in *S. plymuthica* IC1270-primed rice leaf sheath cells following challenge inoculation with conidial suspension of rice blast pathogen. Epifluorescence image of IC1270-induced sheath cells at 24 h post-inoculation; *Ap* appressorium; Scale bar: 20 μm (Courtesy of de Vleeschauwer and Höfte 2007 and with kind permission of BioMed Central, BMC Plant Biology)

network, as induced plants displayed an enhanced susceptibility to *R. solani* and *B. oryzae.* Artificial enhancement of reactive oxygen species (ROS) levels in inoculated leaves faithfully mimicked the opposite effects of IC1270 strain on these two pathogens, suggesting a central role for oxidative events in the IC1270-induced resistance mechanism (de Vleeschauwer et al. 2009).

Serratia marcescens is another rhizobacterial species, capable of inducing systemic resistance in plants against fungal and bacterial plant pathogens. The strain 90–166 elicited induced systemic resistance (ISR) in cucumber against *Pseudomonas syringae* pv. *lachrymans, Colletotrichum orbiculare* and *Erwinia tracheiphila* under field conditions. The ISR development was found to be influenced by bioavailability of iron and ISR was reduced, when iron was made available in planting medium. ISR induced by the strain 90–166 was not very much dependent on salicylic acid (SA), since three mutants of 90–166 lacking SA production were able to induce resistance in cucumber against anthracnose caused by *C. orbiculare* (Press et al. 1997). Seed treatment with the strain 90–166 suppressed anthracnose disease of cucumber, when iron concentrations of the planting mix was decreased by the addition of an iron chelator ethylenediamine [di(o-hydroxyphenyl acetic acid)] (EDDHA). Suppression of the disease by the strain 90–166 was significantly improved. A mutagenesis approach was applied to evaluate the role of siderophore production by 90–166 in ISR. A mutant 90-166-2882, lacking catechol siderophore production, had a transposon insertion in an *entA* homolog. The *entA* mutant

90-166-2282 could not induce resistance to anthracnose, supporting the hypothesis that catechol siderophore produced by the wild-type strain 90–166 is necessary for elicitation of ISR. The *entA* mutant still retained the ability to produce SA, but not the ability to elicit ISR, indicated that catechol rather than SA was required for induction of ISR against cucumber anthracnose disease. The lack of capacity to elicit ISR by mutant strain 90-166-2882 was not attributed to inefficient rhizosphere colonization, since the total root population sizes of the mutant and wild-type strains were similar. The results suggested that catechol siderophore biosynthesis genes in *S. marcescens* 90–166 might be associated with ISR (Press et al. 2001). The ability of chitinolytic *S. marcescens* GPS5 with and without chitin supplementation (1 % colloidal chitin) to activate defense-related enzymes in groundnut leaves was assessed, following challenge inoculation with *Phaeoisariopsis personata,* causing late leaf spot disease. Chitin supplementation reduced the lesion frequently by 64 % as against 23 % with chitin supplementation. Chitin-supplemented GPS strain was much more effective in the activation of defense-related enzymes and this enhanced enzyme activities continued up to 13 days after pathogen inoculation (Kishore et al. 2005a, b).

The mechanism of antagonistic activity of three strains of *Serratia marcescens* (CFFSUR-B2, CFFSUR-B3 and CFFSUR B4) against the fruit anthracnose pathogen *Colletotrichum gloeosporioides* was studied. All the three strains showed similar pattern of antagonistic activities with differences in their extent of inhibition of mycelial growth (>40 %) and germination of conidia (81–89 %) of *C. gloeosporioides*. The bacterial strains produced prodigiosin and chitinases in oilseed-based media (peanut, sesame, soybean or castor bean) and Luria-Bertani medium. Production of prodigiosin reached the maximum (40 µg/ml) level in the peanut-based medium, while cultivation in soybean-based medium induced the highest quantity of chitinase (56 units/ml) in all strains of the BCA. Prodigiosin production was not influenced by changes in pH of the medium. The strains of *S. marcescens* used in this investigation showed the ability of adapting to tropical climates (Gutiérrez-Román et al. 2012).

5.1.7 Pantoea *sp.*

Biological suppression of a plant disease with *Pantoea agglomerans* (earlier known as *Erwinia herbicola*) was first attempted in the 1930s (Beer et al. 1984). *P. agglomerans* (*Pa*) is a Gram-negative bacterium included in the family Enterobacteriaceae. *Pa* is primarily a plant epiphyte, but commonly found in diverse niches including aquatic environment, soil and sediments. Some strains are capable of inducing human ailments. Fire blight disease of apple and pear caused by *Erwinia amylovora* is a destructive disease. Many strains of *Pa* have been developed as commercial products for application as biocontrol agents against fire blight and other crops diseases caused by bacterial pathogens (Rezzonico et al. 2009). Biological control focused primarily on suppression of epiphytic growth phase of *E. amylovora* on

blossoms prior to infection and epiphytic growth. Strains of *P. agglomerans* were evaluated as potential biocontrol agents against *E. amylovora*, in part, due to their ability to produce antibiotics inhibitory to the pathogen in synthetic culture media. The antibiotics produced by the strains of *P. agglomerans* were identified as herbicolins (Ishmaru et al. 1988), pantocins (Brady et al. 1999; Wright et al. 2001) or microcins (Vanneste et al. 1998). Some strains of *P. agglomerans* could produce single antibiotics, while some produced multiple compounds that suppressed the pathogen development. The genes encoding for antibiotic biosynthesis were either localized in the chromosomes and/or on plasmids. Pear juice contains several amino acids including arginine and proline which can reverse inhibition of *E. amylovora* by the antibiotics produced by *P. agglomerans.* The laboratory assay has limitations in that strains that suppress fire blight through competitive exclusion of the pathogen from sites and nutrients on stigmas might be lost, because of their inability to inhibit the pathogen growth.

The role of antibiotics produced by strains of *Pantoea agglomerans* in fire blight disease suppression was investigated by employing transformed bacterial species and mutants deficient in the production of antibiotics. *Escherichia coli* was transformed with a 12-kb plasmid isolated from *E. herbicola*. The transformants that were able to produce antibiotics, inhibited the growth of the pathogen in the culture and symptoms development on inoculated hawthorn blossoms. In contrast, the wild-type strain did not inhibit the growth of and induction of symptoms by the pathogen (El-Masry et al. 1997). In the another approach to study the role of antibiotics in disease suppression, mutants of the strain Eh318 deficient in pantocin A, pantocin B or both antibiotics were generated. Mutants of Eh318 deficient in the production of pantocin A or pantocin B were equally effective in reducing infection by *E. amylovora,* as the wild type strain in the greenhouse experiments. On the other hand, the mutant deficient in the production of both antibiotics were less effective in suppressing the development of fire blight disease, indicating that the strain Eh318 might suppress the pathogen development through other mechanism (s) in addition to antibiosis (Wright and Beer 1996; Wright et al. 2001).

The effectiveness of secondary metabolites produced by *Pantoea agglomerans* in suppressing fire blight disease caused by *Erwinia amylovora* was assessed in apple or pear orchards. The population dynamics and disease suppression with *P. agglomerans* Eh252, a strain that could produce a single antibiotic, were compared with its near-isogenic antibiotic deficient derivative, strain 10:12. Water or suspensions of Eh 252 or 10:12 (1×10^8 CFU/ml) were applied at 30 and 70 % bloom to pear or apple trees. Aqueous suspensions of freeze-dried cells of *E. amylovora* (3×10^5 CFU/ml) were applied at full bloom. Population sizes of Eh252 or 10:12 on pear blossoms were estimated by spreading dilutions of blossom washes on culture media. Average population sizes of Eh252 and 10:12 on blossoms ranged from 10^5 to 10^7 CFU. The antibiotic deficient strain 10:12 was generally less effective than Eh 252 in suppressing fire blight. However, it significantly reduced the incidence of fire blight in four of seven experiments, compared with inoculated water-treated controls. Eh 252 significantly decreased the incidence of fire blight in six of seven field trials, compared with the incidence on plants treated with 10:12 mutant and

Table 5.10 Effect of treatment with strain Eh252, mutant 10:12 and commercial antibiotics on incidence of fire blight disease on pear cultivars during 1998–2000 (Stockwell et al. 2002)

Year/cultivars		Treatments				
		Water	Eh 252	10: 12	Oxytetra-cycline	Strepto-mycin
1998	Bartlett	1.00a (188)	0.32c	0.65b	1.65a	0.41c
1999	Bartlett	1.00a (160)	0.36bc	0.48b	0.51b	0.12c
	Bosc	1.00a (29)	0.88a	0.90a	NT	0.54b
	Bartlett (Medford)	1.00a (63)	0.12d	0.31c	0.62b	0.06d
2000	Bartlett	1.00a (166)	0.59b	0.78ab	0.69b	0.17c
	Bosc	1.00a (204)	0.51a	0.98a	NT	0.08c
	Rome beauty	1.00a (50)	0.16c	0.50bc	0.66b	0.32bc
	Pooled data	1.00a	0.45d	0.70c	0.84b	0.25e

Figures for each of the pear cultivars in each year (*horizontal*) with same letter are not statistically significant (P = 0.05) according to Fischer's protected least difference; number in parentheses are the average number of fire blight strikes on water-treated (*control*) trees
NT not tested

water control. Overall the strain Eh 252 reduced the incidence of fire blight by 55 ± 8 %, 10:12 by 30 ± 6 %, streptomycin by 75 ± 4 % and oxytetracycline by 16 ± 14 %. The moderate level of effectiveness of strain 10:12 compared with water control, indicated that other mechanisms such as competitive exclusion or habitat modification might also contribute to suppression of fire blight disease. Disease suppression by Eh 252 exceeded the level that was provided by oxytetracycline, but it was similar to that provided by streptomycin in several field trials (Table 5.10). The results indicated that antibiosis was an important mechanism of biocontrol activity of *P. agglomerans* Eh 252 against *E. amylovora*, causal agent of fire blight disease of pear and apple (Stockwell et al. 2002).

Pantoea agglomerans strain E325 was selected through screening, based on suppression of *Erwinia amylovora* on flower stigmas. In order to have an insight into the underlying mechanism of biocontrol activity of strain E325, bacterial modification of pH was evaluated by analyzing exudates extracted from 'Gala' apple stigmas. Under ideal laboratory conditions for bacterial colonization of flowers, *P. agglomerans* strain E325 was able to reduce the pH on stigmas to levels that could reduce growth of *E. amylovora.* In contrast, an increase in pH occurred on flowers inoculated with the pathogen alone. Similar trend of enhancement of pH in pathogen-inoculated flowers and decrease of pH in antagonist-treated flowers was also observed (Fig. 5.11). In addition under low-phosphate and low-pH conditions, an antibacterial product of E325 with high specificity to *E. amylovora* was able to suppress the pathogen growth at low concentrations. The antagonistic activity was heat stable and it was not lost due to treatment with amino acids, iron or enzymes known to inactivate antibiotics effective against *P. agglomerans.* The results suggest that the strain E325 might suppress the development of the fire blight pathogen, not only by competing for nutrients on the stigma and producing antibiotics, but also by modifying flower stigma environment (Pusey et al. 2008). The mechanism of biocontrol activity of *Pantoea agglomerans* strain E325 against the fire blight

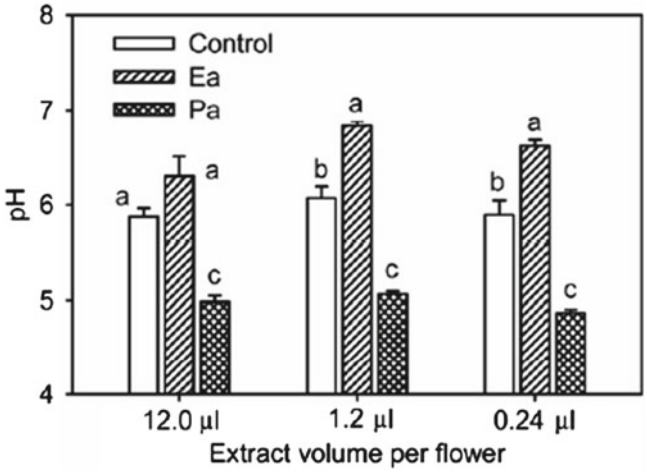

Fig. 5.11 Bacterial modification of pH of exudates extracted from 'Gala' apple stigmas treated with *Pantoea agglomerans* E25 (*Pa*) and inoculated with *Erwinia amylovora* (Ea). *Vertical lines* represent standard error within each bar group; means with same letter are not significantly different according to the least significant difference ($P \leq 0.05$) (Courtesy of Pusey et al. 2008 and with kind permission of The American Phytopathological Society, MN, USA)

pathogen *Erwinia amylovora* was studied. Antibiosis was evaluated as a mode of antagonism on flower stigmas using two antibiosis-deficient mutants. Mutants were tested against *E. amylovora* on stigmas of detached flowers of crab apple (*Malus mandshurica*) in growth chambers and apple (*Malus domestica*) in the orchard. Epiphytic fitness of the antibiosis-negative mutants was similar or greater than the wild-type strain as reflected by relative area under population curve (RAUPC). Both mutants were less effective in inhibiting pathogen development in in vitro assays and under orchard conditions. The mutants and the parent strain showed distinct differences in the colony morphology. However, E325 and the mutants induced similar level of decrease in pH in a broth medium, indicating acidification of the medium which was not related to antibiosis. The results provided evidence for the involvement of antibiosis as the mechanism of the biocontrol activity of *P. agglomerans* E325 against *E. amylovora* (Pusey et al. 2011).

The mechanism of biocontrol activity of *Pantoea agglomerans* strain CPA-2 effective against the postharvest pathogens *Penicillium digitatum* and *P. italicum* respectively causing green mold and blue mold diseases of citrus, was studied. The strain CPA-2 did not produce antibiotics or chitinolytic enzymes that could account for the suppression of the mold diseases. The antagonist did not induce the activation of defense-related enzymes PAL and PO in the orange peel at different periods after inoculation with the antagonist and/or the pathogen. *P. agglomerans* could effectively suppress the pathogens, only when it was in close contact with the pathogens. Competition for nutrients was assessed, using tissue culture plates with cylinder inserts which allowed competition for nutrients to be studied without competition for space. The physical contact between pathogen and the bacterial strain was

avoided by this setup. The germination of conidia of *Penicillium* spp. was substantially decreased by the antagonist, when the diluted orange peel extract or diluted potato extract or diluted potato-dextrose was the nutrient source. Direct contact between the pathogen and bacteria resulted in complete inhibition of conidial germination. The results indicated that although the competition for nutrients might be one mechanism of biocontrol activity of CPA-2, physical contact between the pathogen and the BCA was important for greater effectiveness of the biocontrol activity against the mold pathogens infecting citrus fruits (Poppe et al. 2003). The ability of *Pantoea agglomerans* strain CPA-2 to elicit resistance in oranges against *Penicillium digitatum* was investigated, by determining the generation of hydrogen peroxide (H_2O_2) and changes in the activities of superoxide dismutase (SOD) and catalase (CAT) activities at different periods after inoculation of oranges with the pathogen. At 3 days after inoculation, CPA-2 treated fruit showed an accumulation of H_2O_2. The activities of SOD and CAT also registered increases in oranges treated with CPA-2 strain, while infected fruit showed sharp decreases in the levels of all three enzymes. The results suggested that as a response to pathogen infection, H_2O_2 production, as well as activities of SOD and CAT, were suppressed, whereas the BCA strain accelerated the H_2O_2 generation and enzyme activities involved in the activation of disease resistant systems. The CPA-2 strain appeared to have multiple mechanisms of biocontrol activity against green mold pathogen *P. digitatum* infecting stored orange fruits (Torres et al. 2011).

The biocontrol potential of another strain 59–4 of *Pantoea agglomerans* in suppressing the development of green and blue mold diseases in mandarin oranges was assessed. The strain Pa 59–4 was isolated from soil samples from different locations in Korea. The concentration of cells of Pa 59–4 required for the inhibition of *Penicillium digitatum* (causing green mold) was less (10^5–10^6 cells/ml) than the concentration (10^8–10^9 cells/ml) required for inhibition of *P. italicum* (causing blue mold). Pa 59–4 suppressed infection by green and blue mold pathogens on wounded mandarins to the extent of 85–90 % and 75–80 % respectively. Higher concentrations of Pa 5904 provided better protection, indicating the dosage effect of the BCA. The ability to effectively colonize wounds on the peels of citrus fruit is important for the survival of Pa 59–4, because its antagonistic action was primarily based on physical contact with the pathogen in infection site and also on competition for nutrients. Pa 59–4 in wounded mandarin fruits increased more than ten times during 24 h of incubation at 20 °C. The results indicated that competition for nutrient might play a role in the inhibition of conidial germination, as the strain Pa 59–4 could not inhibit the mycelial growth of *Penicillium* spp. in plate confrontation assays (Yu et al. 2010).

The inconsistency of the performance of biocontrol agents to provide effective fire blight disease suppression may be due to instability of antagonist populations, environmental conditions or host physiological changes associated with flower development and senescence. Knowledge on these factors will be useful for improving the efficacy and consistency of performance of the bacterial biocontrol agents like *Pantoea agglomerans* strain E325 and *Pseudomonas fluorescens* A506 that have been reported to be effective against *Erwinia amylovora*. The flower stigma is of considerable

importance as the site of pathogen establishment and the site where antagonist adaptation is considered to be critical for effective biocontrol. Growth of *E. amylovora* on flower stigma is dependent on temperature as revealed by forecasting systems (Billing 2000). The temperature affects the duration of stigma receptivity to pollen and the stigma senescence is accelerated after pollination. Generally the pathogen reached higher population levels than the antagonists at temperatures from 20 to 32 °C. The populations of *P. agglomerans* strains C9-1 and E325 attained relatively high levels at these temperatures (Pusey and Curry 2004). The difference in temperature optima of strains A506 and C9-1 was considered to be a mode by which these two antagonists could complement each other when applied as a mixture. The dominant strain tended to be A506, during relatively low temperature years and C9-1 in high temperature years (Johnson and Stockwell 2000). Detached Manchurian crab apple flowers were inoculated with *E. amylovora* and subjected to a range of constant temperatures or various fluctuating temperature regimes. Maximum stigma age supporting bacterial multiplication decreased, as temperature increased and was reduced by pollination. Stigmas were receptive to bacteria at ages older than the age reported earlier, possibly because of less interference from indigenous organisms. The results indicated that antagonists could not grow on relatively old stigmas conducive to the pathogen. It may be possible to select antagonists that can develop on old stigmas rapidly to exclude the pathogen from such ecological niche (Pusey and Curry 2004).

5.1.8 Agrobacterium *spp.*

The mechanism of suppression of crown gall disease caused by *Agrobacterium tumefaciens* by the non-pathogenic *Agrobacterium rhizogenes* (*A. radiobacter*) strain K84 is unique. Differentiation of the pathogen and the biocontrol agent is based only on the presence of the plasmid with T-DNA responsible for infection in the pathogen and its absence in the BCA. On the other hand, the BCA possesses another plasmid responsible for the production of a bacteriocin termed agrocin 84 which suppresses the development of *A. tumefaciens* and gall formation in susceptible host plants (Kerr 1980). The strain K84 was not found to be effective against all strains of *A. tumefaciens*. *A. vitis* causing crown gall disease of grapevine was not sensitive to agrocin 84. Agrocin 84 targeted a tRNA synthetase in tumorigenic *Agrobacterium* strains (Reader et al. 2005). *Agrobacterium tumefaciens* causes crown gall disease in large number of host plants including crop plants. *Agrobacterium* strains D286, HLB2 and K1026 produced agrocins effective against nopaline strains of *A. tumefaciens*. Strain K84 was effective only against nopaline strains of *A. tumefaciens,* but not against octopine strains causing crown galls on grapevine. A nonpathogenic strain of *A.vitis* E26 was found to be effective against *A. vitis* causing crown galls on grapevine and also against *A. tumefaciens* and *A. radiobacter* infecting peach and cherry respectively. An antibacterial compound Ar26 with MW 76-kDa was isolated from the culture supernatant of the strain E26. The compound Ar26 strongly inhibited the growth of *A. vitis* MI3-2 and *A. tumefaciens* CY4 on culture plates (Wang et al. 2003).

Another strain of *Agrobacterium vitis* F2/5 inhibited the growth of most tumorigenic strains of *A. vitis* in vitro and inhibited crown gall development on grapevine in stem-wounding experiments in the greenhouse. The strain F2/5 also produced a bacteriocin and effectively inhibited tumor formation at wound sites on grapevine stems inoculated with a tumorigenic strain of *A. vitis* (Burr and Reid 1993). Suppression of tumor development in grapevine by the strain F2/5 was not dependent on the production of agrocin, since the agrocin-minus mutant of the BCA was able to reduce gall production as effectively as the wild-type strain (Burr et al. 1997). A new strain K1026 was constructed by using recombinant DNA techniques. The new strain was identical to K84 apart from 5.9-kb deletion overlapping the Tra region of pAgK84. The strain K1026 was unable to transfer its mutant agrocin 84 plasmid, designated pAgK1026 to other agrobacteria, but it remained antagonistic to strains sensitive to agrocin (Jones and Kerr 1989). Agrocin mutants of nontumorigenic *A. vitis* strain F2/5 suppressed grape crown gall development as the wild-type strain, indicating agrocin was not the major factor in the mechanism of biological disease suppression. Relative levels of attachment to grape cells by tumorigenic and biocontrol strains were measured. Attachment of tumorigenic strains (CG49 and K306) and biocontrol strains (F2/5 and agrocin mutant 1077) was frequently reduced, when mixtures of the strains were applied. However, high populations (10^3–10^5 CFU/ml) of all strains attached following mixed inoculation, suggesting that competition for attachment sites was not a factor in the mechanism of biocontrol activity of the BCA strains tested. Transfer of T-DNA to grape by CG49 was prevented or greatly inhibited in the presence of F2/5 or 1077 as measured by expression of the GUS reporter gene. Sonicated and autoclaved preparation of F2/5 and 1077 did not suppress crown gall development in grapevine or inhibit the T-DNA transfer to grapevine cells. Suppression of crown gall disease by the strain F2/5 was specific to grapevine, since it was ineffective against gall formations on tomato and sunflower (Burr et al. 1997).

A nonpathogenic *Agrobacterium vitis* strain VAR 03–1 capable of producing a bacteriocin, greatly inhibited tumor formation on stems of tomato and grapevine seedlings caused by several tumorigenic *A. vitis* strains. When roots of grapevine, rose and tomato were soaked in a cell suspension of VAR 03–1, before planting in soil infested with tumorigenic *A. vitis, A. rhizogenes* and *A. tumefaciens* respectively, the antagonist treatment significantly reduced the number of plants with tumors and disease severity in the plant species tested. The inhibitory effects of treatment with VAR 03–1 on grapevine was more effective than that of treatment with K84, whereas the effects of treatment with both BCA strains on rose and tomato were almost similar. The strain VAR 03–1 was bacteriocinogenic, producing a halo of inhibition against all the three species of *Agrobacterium*. Under field conditions, the strain VAR 03–1 was highly effective in reducing the incidence of the crown gall disease in grapevine. This strain could establish populations averaging 10^6 CFU/g of roots in the rhizosphere of grapevine and persisted on roots for 2 years. The results indicated that the strain VAR 03–1 could be applied for effective control of crown gall diseases caused by *Agrobacterium* spp. (Kawaguchi et al. 2008).

5.1.9 Myxobacteria

Myxobacteria are Gram-negative, unicellular bacteria with rod-shaped vegetative cells. They are soil dwelling, gliding bacteria that form fruiting bodies containing myxospores. Thick-walled myxospores are resistant to desiccation, high temperature and UV-radiation and hence, they are responsible for the survival of myxobacteria. These bacteria specialize in biodegradation of biomacromolecules and are considered as micropredators, because the antibiotics and/or enzymes produced by them can kill microorganisms and lyse cells from which bio-macromolecules are then scavenged (Dawid 2000). Myxobacteria produce many different classes of antibiotics, cell wall-degrading enzymes, lipases, nucleases, polysaccharidases and proteases. The antibiotic pyrrolnitrin produced by myxobacterial species is of agricultural importance (Rosenberg and Varon 1984).

Myxococcus spp. were isolated from soils in organic and conventionally managed strawberry production and transplant fields that were not treated with methyl bromide or chloropicrin which virtually eliminated these bacteria. Six *Myxococcus* spp. were evaluated for the antagonistic activity against fungal pathogens and two well known fungal biocontrol agents *Gliocladium virens* and *Trichoderma viride*. The pathogen *Phytophthora capsici*, *Pythium ultimum, Rhizoctonia* spp., *Sclerotinia minor* and the BCA *T. viride* were entirely inhibited by all of the *Myxococcus* spp. tested. *M. coralloides* inhibited the growth of *Cylindrocarpon* spp. *Fusarium oxysporum* f.sp. *apii, Verticillium albo-atrum* and *V. dahliae,* in addition to the fungal pathogens that were inhibited by other *Myxococcus* spp. The BCA *Pseudomonas fluorescens* CHA0 was protected by secondary metabolite production regulated by *gacS* from lysis by myxobacteria. Phenazine antibiotics were responsible for the protection of *P. aureofaciens* strain 30–84 from lysis by the myxobacteria (Bull et al. 2002). *Myxococcus* spp. strain KYC 1126 inhibited completely the spore germination of *Botrytis cinerea*, *Colletotrichum acutatum* and *Magnaporthe grisea*. The activity of the bioactive compounds present in the culture supernatant was found to be fungicidal, but the liquid culture filtrate of KYC 1126 did not affect protoplast reversion in *C. acutatum.* Application of culture filtrate on hot pepper plants reduced the incidence of anthracnose disease significantly under both greenhouse and field conditions (Kim and Yun 2011).

5.1.10 Achromobacter

Achromobacter spp. is a Gram-negative bacteria and catalase-positive. It can be obtained frequently from the rhizosphere. *A. xylosoxydans* isolate MM1 was isolated from a *Fusarium*-suppressive soil in Italy. The biocontrol potential of the isolate MM1 was assessed against Fusarium wilt disease of tomato caused by *F. oxysporum* f.sp. *lycopersici.* The bacterial strain was applied by dipping plant roots in bacterial cell suspension (1×10^8 CFU/ml). The disease incidence was

reduced by about 50 % under severe disease pressure conditions, while up to 81.3 % infection was recorded in the control treatment. In vitro assays, no chitinolytic activity or antibiotic production by the isolate MM1 could be detected and no inhibition of growth of the pathogen was observed in plate confrontation tests. The isolate produced siderophore in malt extract agar (MEA) medium supplemented with $FeCl_3$. The disease suppressive effect of isolate MM1 in vivo might be related to its ability to produce siderophores that limit the bioavailability of iron to the pathogen. In addition, the possibility of *A. xylosoxydans* eliciting induced systemic resistance (ISR) in tomato against the wilt pathogen was also suggested by Moretti et al. (2008).

5.1.11 Actinomycetes

Actinomycetes are generally saprophytic, soil dwelling bacteria, spending majority of their life cycle as spores. They represent a high proportion of the soil microbial biomass and have the potential to produce a wide range of antibiotics and extracellular enzymes. Several strains of actinomycetes are able to protect crop plants against diseases caused by microbial pathogens and some of them promote growth of plants too. The antibiotic geldanamycin produced by *Streptomyces hygroscopicus* var. *geldanus* suppressed the development of *Rhizoctonia solani* causative agent of Rhizoctonia root rot disease of pea. The methanol extracts of soils in which the antagonist was incubated for two or more days, inhibited the growth of *R. solani*. The concentration of geldanamycin was 88 µg/g of soil at 7 days after incubation. The period of incubation necessary for antibiotic production and disease suppressed matched with each other (Rothrock and Gottlieb 1984). In a later study, *S. hygroscopicus* var. *geldanus* strain EF-76 was shown to protect potato against common scab disease, indicating the vital role of geldanamycin production by this BCA strain in its ability to control the disease (Beauséjour et al. 2001).

Streptomyces diastatochromogenes strain PonSSII produced antibiotics inhibitory to pathogenic strains of *S. scabies* causing potato scab disease and reduced the number lesions formed on tubers significantly. The inhibitory effect was directly correlated to the more vigorous growth and larger inhibition zones of the strain PonSSII against pathogenic strains, compared with another BCA strain PonR in in vitro tests. Production of antibiotic by the strain PonSSII could be stimulated or repressed by the presence of other *Streptomyces* strains including pathogenic strains. It appeared that interspecies/strain communication could be of significance in pathogen suppression (Becker et al. 1997). *S. violaceusniger* strain YCED9, isolated from the rhizosphere, suppressed the development of damping-off disease of lettuce caused by *Pythium ultimum* (Crawford et al. 1993). The strain YCED9 produced nigericin, geldanamycin and a fungicidal complex of polyene-like compounds designated AFA (anti-*Fusarium* activity) that included guanidylfungin A. Production of these antimicrobial compounds was significantly influenced by the composition of media on which the strain YCED9 was grown. The actinobacteria strain provided effective

protection against the turfgrass pathogens *Colletotrichum graminicola, Sclerotinia homeocarpa, Gaeumannomyces graminis* and *Rhizoctonia solani* (Trejo-Estrada et al. 1998). *Streptomyces* spp. isolated from the soil was evaluated for its ability to suppress the growth of *Sclerotium rolfsii* and development of damping-off disease in sugar beet. Among the four isolates, J-2 was the most efficient in inhibiting the mycelial growth of *S. rolfsii*. Inhibition of sclerotial germination occurred in soils treated with biomass inoculum and culture filtrate of the isolate J-2, the percentage of inhibition being 93 and 88 % respectively. Seed treatment with the isolate J-2 resulted in significant reduction in disease severity in the seedlings. The results suggested that antibiosis might be the mechanism of biocontrol activity of *Streptomyces* spp. against *S. rolfsii* (Errakhi et al. 2007).

Streptomyces rimosus MY02, isolated from soil, produced antifungal metabolites in the culture medium. An antifungal metabolite SN06 was extracted from the culture of the strain MY02 using n-butanol and purified by silica gel column chromatography. The minimum concentration of SN06 inhibitory to *Fusarium oxysporum* f.sp. *cucumerinum* was 12.5 μg/ml. Using light microscope, the changes in morphology of *F. oxysporum* f.sp. *cucumerinum* treated with SN06 were determined. Some of the hyphal cells began to dilate and formed some strings of heads. The cytoplasm oozed out of the cells and most of hyphal cells were empty. The hyphae were cleaved into many segments and then collapsed after 48 h. Damage to mycelial cell membrane and leakage of nucleic acid from the damaged fungal cells were indicated by scanning, using ultraviolet spectrophotometer and absorption at 260 nm (Liu et al. 2009a, b). The *Streptomyces* strain (AS1) inhibited both germination and growth of *Aspergillus flavus* producing the mycotoxin aflatoxin B_1(AFB_1) both directly or via secondary metabolites. The core extracts of AS1 metabolites at 50 and 100 ppm entirely inhibited germination of conidia of *A. flavus* over 48 h. The metabolites extractable with ethylacetate were the most effective. Concentrations of 50, 200 and 500 ppm of AS1 metabolites significantly inhibited the populations of *A. flavus* on stored peanuts at two water stress levels (0.90, 0.93 a_w) at 25 °C over 14-day storage periods. The amounts of AFB_1 produced by *A. flavus* on peanuts were also reduced by application of AS1 metabolites, indicating the inhibitory effects of metabolites on the population of *A. flavus* and the amount of mycotoxin produced (Sultan and Magan 2011).

Streptomyces hygroscopicus strain SRA14 was assessed for its potential to produce extracellular hydrolytic enzymes that may be involved in its biocontrol activity against *Colletotrichum gloeosporioides* and *Sclerotium rolfsii.* Production of extracellular chitinolytic and β-1,3-glucanolytic enzymes by the strain SRA14 was determined at different growth phases. The level of chitinase was sharply increased during the exponential phase and dramatically declined, when the cells entered the stationary phase. The highest level of β-1,3-glucanase was produced at 2 days after incubation period and decreased slightly during the stationary phase. Inhibition of mycelial growth of the fungal pathogens *C. gleosporioides* and *S. rolfsii* was due to the activities of chitinase and β-1,3-glucanase secreted by the strain SRA 14 (Fig. 5.12). Observations under light microscope to determine the changes in the fungal morphology, following exposure to cell-free culture

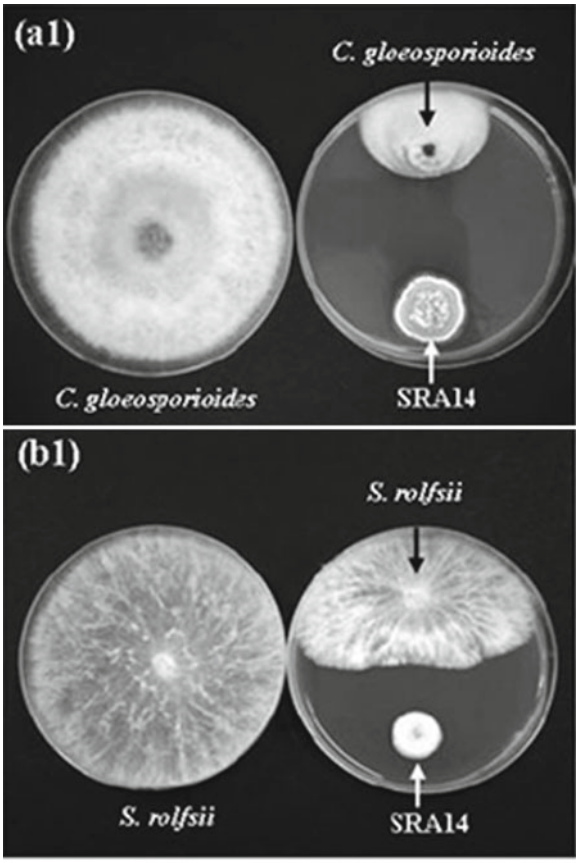

Fig. 5.12 Inhibition of mycelial growth of *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* by *Streptomyces hygroscopicus* SRA14 in dual culture plate assay (Courtesy of Prapagdee et al. 2008 and with kind permission of Ivyspring International Publisher)

filtrate of SRA14 revealed hyphal swelling, distortion and cytoplasm aggregation, indicating adverse effects of extracellular metabolites on the pathogens growth (Prapagdee et al. 2008).

The biocontrol agents and the phytopathogens have to compete with each other for nutrients and space available in the habitat. Some actinomycetes are able colonize inside the roots and other plant tissues and they are known as endophytic actinomycetes. *Streptomyces griseorubiginosus* strain S96 colonized healthy roots of banana and its biocontrol potential against *Fusarium oxysporum* f.sp. *cubense* (*Foc*) race 4, the causal agent of Panama (Fusarium) wilt disease was assessed. The antagonism of strain S96 against *Foc* was conditioned by the bioavailability of iron. There was no antagonism, when the pathogen and BCA were inoculated onto banana tissue extract (BTE) medium containing more than 0.05 % $FeC1_3$. But the BCA was antagonistic in the same medium containing less than 0.05 % $FeC1_3$, indicating that iron-limiting condition favored the antagonistic activity of the strain S96. In addition, the antagonism of strain 96 was lost, when FeCl3 was introduced into the inhibition zone. In vivo biocontrol assays demonstrated that the disease severity

index (DSI) was significantly reduced and mean fresh weight increased in plantlets treated with strain S96, compared to untreated control plantlets (Cao et al. 2005).

Rhizosphere competence of the biocontrol agents is one of the critical requisites for effective suppression of soilborne pathogens. *Streptomyces griseoviridis* has been reported to be a good rhizosphere colonizer. *S. griseoviridis* was found to be an efficient antagonist of fungal pathogens, causing diseases such as Fusarium wilt disease of carnation, damping-off disease of Brassica and root rot disease of cucumber (Tahvonen and Lahdenpera 1988). The mechanism of root colonization by *Streptomyces* spp. may be affected by different factors. Various plant species produce different types and concentration of compounds in their root exudates which may significantly influence root colonization by various kinds of microorganisms including actinomycetes. The potential of *S. griseoviridis* applied as seed treatment for barley and spring wheat was assessed against foot rot disease. Yields of wheat were increased to a greater extent by seed treatment with *S. griseoviridis* than that of barley, probably because the BCA colonized wheat roots more effectively (Weller 1988). *Streptomyces globisporus* JK-1 was evaluated for its antifungal activity against the postharvest pathogen *Penicillium italicum* causing blue mold disease of citrus fruits. The strain JK-1 inhibited the spore germination and mycelial growth of *P. italicum*. Sporulation and disease incidence on pathogen-inoculated fruit were suppressed in the presence of volatiles produced by the strain JK-1. Treatment of naturally infected citrus fruit with the BCA reduced the disease incidence from 25 to 7.5 %. Scanning electron microscopic observations revealed inhibition of spore germination on the Shatang Mandarin orange and abnormal morphology of conidiophores and hyphae exposed to the volatiles of JK-1. Gas chromatography/mass spectrophotometric analyses indicated the presence of 41 organic compounds in the volatiles of JK-1. These volatiles showed inhibitory effect to varying degrees. Dimethyl disulfide or dimethyl trisulfide showed antifungal activity in in vitro assays and also effectively reduced the disease incidence on Shatang Mandarin, when applied at a concentration of 100 µl/l of air space in treatment containers. Acetophenone was effective in suppressing disease development only at higher concentration (1,000 µl/l). The results indicated the potential of the volatiles produced by *S. globisporus* JK-1 for effective suppression of development of blue mold disease of citrus caused by *P. digitatum* (Li et al. 2010).

The effect of volatiles produced by *Streptomyces globisporus* JK-1 on the development of *Botrytis cinerea,* causing gray mold of tomato fruit was investigated. *S. globisporus* was grown on autoclaved wheat seed. The mycelial growth, conidial germination and sporulation of *B. cinerea* and *Sclerotinia sclerotiorum* were suppressed by the volatile compounds produced by the strain JK-1. Incidence and severity of gray mold disease on wound-inoculated tomato fruit were inhibited, when fumigated with 120 g wheat seed culture of JK-1 per liter of air space in treatment containers. Scanning microscopy was used to follow the infection process of *B. cinerea* on tomato fruit. Inhibition of conidial germination and appressorial formation on tomato fruit were observed as well as abnormal morphology of appressoria and conidia. Using the vital stain fluorescein diacetate (FDA) and propidium iodide (PI),

the viability of conidia, after exposure of the disease lesions to the volatiles, was assessed. Reduction in conidial viability varied from 46 to 48 % depending on the concentration of the BCA culture. Transmission electron microscopy (TEM) of fumigated and untreated *B. cinerea* showed excessive vesiculation or thickened cell walls in exposed conidia and increased vesiculation or strong retraction of plasma membrane in exposed hyphae. The results indicated that the mode of action of *S. globisporus* JK-1 could be antagonism, due to the fumigant action of the volatiles produced by the BCA which has the potential for the control of postharvest gray mold disease of tomato fruits (Li et al. 2012).

The efficacy of native *Streptomyces* isolates C and S2 was evaluated for the control of fungal pathogens *Rhizoctonia solani* AG-2, *Fusarium solani* and *Phytophthora drechsleri* associated with sugar beet root rot disease. The isolate C inhibited the mycelial growth of *R. solani*, *F. solani* and *P. drechsleri* by 45, 53 % and 26 % respectively. Incorporation of NaCl into the medium enhanced the biocontrol activity of soluble and volatile compounds of the isolate C and S2. The inhibition percentages of all the pathogens were increased by NaCl addition to the medium. The mechanism of antagonism of *Streptomyces* isolates C and S2 was investigated by analyzing the activities of protease, chitinase, β-glucanase, cellulase, lipase and amylase. Biosynthesis of siderophore by both isolates was also detected. Production of siderophore and activity of protease and amylase by both isolates were increased after adding salt. In contrast, chitinase activity decreased significantly. Production of SA, β-1,3-glucanase and lipase by isolate S2 and biosynthesis of cellulase by isolate C were observed in the presence and absence of NaCl. Soil treatment with *Streptomyces* isolate C reduced the incidence of root rot disease of sugar beet caused by the fungal pathogens. The results indicated that these two isolates of *Streptomyces* could effectively suppress the development of sugar beet root rot disease especially in saline soils (Karim et al. 2012).

The mechanism of biocontrol activity of *Bacillus subtilis* CPA-8 with ability to suppress the development of *Monilinia laxa* and *M. fructicola* infecting peach fruit was investigated. The cell-free supernatants and butanolic extracts of the cultures of the strain CPA-8 showed strong antifungal activities against both fungal pathogens. The presence of fengycin, iturin and surfactin lipopeptides in the butanolic extracts from cell-free supernatants were identified by employing thin layer chromatography (TLC), indicating that antibiosis could be a major factor involved in the biocontrol activity of CPA-8. Strong antifungal activity could be linked to fengycin lipopeptides, as indicated by the results of TLC-bioautography analysis. The transformants from CPA-eight lacking fengycin had reduced or suppressed antifungal activity. Pathogenicity experiments confirmed that fengycin-defective mutants and their cell-free supernatants lost their ability to suppress the development of peach brown rot disease, compared with wild-type CPA-8 strain or Serenade Max®, a commercial product based on *B. subtilis*. However, the fengycin-deficient mutant survived in wounds in peach fruit equally well as the wild-type strain. The results showed that the biocontrol activity of *B. subtilis* CPA-8 depended primarily on its ability to produce fengycin-like lipopeptides for the control of brown rot disease of peach fruits (Yánez-Mendizábal et al. 2012).

Appendix 5.1: Visualization of Effects of the Metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) of *Pseudomonas* spp. on Fungal Pathogen Using Confocal Laser Scanning Microscope (CLSM) (Islam and Fukushi 2010)

A. Preparation of fungal hyphal cells for visualization

i. Grow the fungal pathogen (*Aphanomyces cochlioides*) on potato dextrose agar (PDA) at room temperature (25 °C); cut out agar disks (6 mm diameter) from the growing edges; place the disk individually 30 cm apart from the colony of bacterial biocontrol agent (*Pseudomonas fluorescens* ECO-001) in four replicates: prepare control plates without the BCA and incubate at 25 °C for 5 days in the dark

ii. Harvest the fungal hyphae, using a sterile corkborer (6 mm diameter) from the colony edges growing toward the BCA colonies for observations under confocal laser scanning microscope (CLSM)

iii. Prepare different concentrations of DAPG and latrunculin B (0.1, 0.5, 1.0 and 5.0 μg) in acetone; place the solutions on individual sterile paper disks (8 mm diameter × 1.5 mm thickness) (Advantec Toyo, Japan) and dry the disks by evaporating acetone under vacuum

iv. Place the disks 2 cm apart in petriplates containing PDA inoculated with mycelial plugs (6 mm diameter); cut from the edge actively growing fungal pathogen colony and incubate at 25 °C

B. Preparation of specimen and observation under CLSM

i. Remove mycelial plugs using a sterile cork borer (6 mm diameter) from the edge of the actively growing hyphae of the pathogen paired with bacterial colony or DAPG or latrunculin B-treated paper disks and use plugs removed from untreated plates as control.

ii. Fix the mycelial plugs with 6 % paraformaldehyde in 60 mM 1,4-piperazine- diethanesulfonic acid buffer (Sigma) pH 7.0 with 100 μM MBS (*m*-maleimidobenzoyl *N*-hydroxyl succinimide ester, Pierce) for 30 main at room temperature; rinse three times in a buffer solution and transfer to glass slides for sectioning

iii. Section the upper portion of the agar plug uniformly (0.25 mm thickness) with a sterilized stainless blade

iv. Stain the sections for 30 min in 0.66 μM RP in 60 mM Pipes buffer (pH 7.0); rinse in buffer and mount in 50 % glycerol with 0.1 % p-phenylene-diamine and observe under CLSM

v. Use scan time perframe 1.08 s; to obtain a DIC image and perform averaging at four scans per frame

vi. Repeat the experiment three times with three replicates for each experiment

Appendix 5.2: Assessment of Effect of Phenazines on Microsclerotial Germination of *Verticillium* spp. by Microplate Assay (Debode et al. 2007)

i. Place nylon mesh filters (pore size 41 µm, diameter 25 mm, Millipore, USA) in the wells of 96-well microplate; embed the microsclerotial preparation (20 µl) in the well (approx. 50 microsclerotia/well); add aliquots of 180 µl bacterial suspension (2×10^7 and 2×10^9 CFU/ml); use sterile physiological solution (180 µl) for controls and incubate for 2 days at 24 °C
ii. Retrieve the filters from the wells separately; place them on sterile filter paper under sterile conditions and dry the filters
iii. Place the filters on soil-pectate-tergitol agar (SPTA) plates containing each 50 mg/l of chloramphenicol, tetracycline and streptomycin sulfate and incubate for 10 days
iv. Examine under the dissecting microscope and record the effect on percentage of microsclerotial germination and formation of secondary microsclerotia in different treatments
v. Maintain five replications and repeat the experiment once

Appendix 5.3: Assessment Antagonistic Activity of Bacterial Antagonists Against *Agrobacterium* spp. (Dandurishvili et al. 2010)

A. Dual-culture 'sandwich' assay

i. Fill a petridish with suitable medium seeded with the test antagonist strain and fill another petridish similarly with medium inoculated with the target pathogen strain (overnight culture) at appropriate dilution (10^5 cells/ml)
ii. Join the open plates together and tightly seal with parafilm maintain suitable control (without the antagonistic strain)
iii. Incubate the plates at 28 °C and examine the samples taken at 24-h interval

B. Bioassays for tumorigenicity in the greenhouse

i. Raise the tomato seedling nursery in plastic seed trays for 25–30 days at 25 °C in a growth chamber; transplant the seedlings at 2–3 leaf stage into bigger containers and placed in the greenhouse
ii. Grow the bacterial pathogen and the BCA strains separately in suitable medium for 48 h at 28 °C in a shaker; centrifuge the suspension at 8,000 rpm for 15 min; resuspend the bacterial cells in tap water and adjust the concentration to 10^8 cells/ml
iii. Soak the roots of tomato seedlings in glass vessels containing the BCA cell suspension at 10^7 cells/ml and transplant the seedlings into containers placed in the greenhouse

iv. Make wounds by scratching on the stem surface of seedlings with a needle at two sites (second and third internodes); inject into the wound sites with pathogen suspension (10 µl, 2–5 x 10^8 cells/ml) and treat the control plants similarly only with the pathogen cell suspension
v. Inoculate tomato seedling in the wound sites first with the antagonist cell suspension; after 7 days inoculate the pathogen in the same wounds by injecting the pathogen suspension (10 µl/wound site)
vi. Record the tumor formation in tomato plants inoculated by two different methods and express the disease incidence in terms of tumor fresh weight in different treatments

Appendix 5.4: Assessment of Activities of Enzymes in Papaya Fruits Treated with *Pseudomonas putida* MGY2 (Shi et al. 2011)

A. Phenylalanine-ammonia lyase (PAL) (EC4.3.1.5) activity

i. Perform all steps at 4 °C; homogenize pericarp tissue samples (1 g) in 2 ml of extraction buffer (50 mM Tris–HCl buffer, pH 8.8 containing 15 mM B-mercaptoethanol, 5 mM EDTA, 5 mM ascorbic acid, 1 mM PMSF and 0.15 % PVP (w/v)); filter the homogenate through cheesecloth (four layers); centrifuge at 12,000 g for 20 min at 4 °C and use the supernatant as crude enzyme
ii. Prepare the reaction mixture (3 ml) containing 16 mM L-phenylalanine, 50 mM Tris–HCl buffer (pH 8.8), 3.6 mM NaCl and 0.1 ml of enzyme solution and incubate at 37 °C for 40 min
iii. Stop the reaction by adding 500 µl of 6 mM HCl; centrifuge at 12,000 g for 10 min to precipitate the denatured protein
iv. Record the absorbance at 290 nm using a spectrophotometer before [step (ii)] and after incubation
v. Use cinnamic acid (analytical grade) as standard; one unit of PAL activity is equal to the amount enzyme that produced 1 µmol of cinnamic acid within 1 h
vi. Express the results as µmol of cinnamic acid/mg protein/h

B. Catalase (CAT) (EC1.11.1.6) activity

i. Grind pericarp tissues (5 g) from five papaya fruits in 20 ml of 0.05 M sodium borate buffer (pH 7.0, containing 5 mM mercaptoethanol) with 0.5 g polyvinyl polypyrrolidone (PVPP).
ii. Add 0.2 ml of enzyme preparation to 2.8 ml of 40 mM H_2O_2 (dissolved with 50 mM sodium phosphate buffer, pH 7.0) as a substrate.
iii. Measure the decomposition of H_2O_2 by the decline in absorbance at 240 nm
iv. One unit of CAT is equal to the amount of enzyme per microgram protein that decomposed 1 mol of H_2O_2/min at 30 °C

C. Peroxidase (POD) (EC 1.11.1.7) activity

i. Homogenize the pericarp tissues (1 g) in 4 ml of extraction buffer (0.2 M sodium phosphate buffer, pH 6.4 containing 0.2 % PVP (w/v)) at 4 °C; centrifuge at 12,000 g for 20 min and use the supernatant as crude enzyme
ii. Incubate the mixture containing 0.5 ml enzyme extract and 2 ml of guaiacol substrate (100 mM sodium phosphate, pH 6.4, containing 8 mM guaiacol) for 5 min at 30 °C
iii. Determine the increase in absorbance at 460 nm after adding 1 ml H_2O_2 (24 mM)
iv. One unit of POD activity is equal to the amount of enzyme per microgram protein that increased 0.01 in absorbance at 460 nm in 1 min

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